TRANSCRIPTIONAL REGULATION OF THE MOUSE AND HUMAN INTERLEUKIN-5 GENES IN T-LYMPHOCYTES

By

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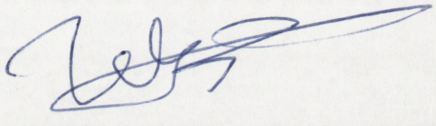
STATEMENT

The research described in this thesis was solely and entirely conducted by the author unless specifically stated otherwise in the text.



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This work is dedicated to my parents who gave me great encouragement and support, especially to my mother who passed away during my PhD; also to my wife, Yafei, for her constant and unfailing support and to my son, Kevin, who despite his youth gave me great support.

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ABSTRACT

The transient transfection systems were established in both mouse (D10W) and human (HSB-2) T cell lines, providing model systems for analysis of the molecular mechanisms regulating IL-5 gene expression. Mouse IL-5 promoter activity was induced 36-fold by stimulation with PMA/cAMP in D10W cells whereas stimulation with PMA/cAMP/ionophore was required for 6-fold induction of the human IL-5 promoter in HSB-2 cells. Both inductions were abolished when the putative transcription factor binding sites for AP-1, Ets/NFAT or GATA in the proximal promoter regions of the respective IL-5 genes were mutated. Transactivation studies showed that the transcription factor AP-1 (c-Jun and c-Fos) strongly activated both the mouse and human IL-5 promoters in the absence of stimulation. In unstimulated cells, AP-1 synergistically transactivated the mouse IL-5 promoter in combination with Ets1 and cooperated with both Ets1 and GATA-3 to activate the human IL-5 promoter. Electrophoretic mobility shift assays showed that recombinant AP-1 and Ets1 proteins specifically bound to their respective sites in the mouse IL-5 promoter. The transactivation activities of the transcription factors were shown to be mediated through the AP-1, Ets/NFAT and GATA binding sites in the proximal promoter region of the respective genes. The transactivation activity of Ets1 plus its unique ability to synergize with AP-1 strongly suggested that Ets1 rather than Elf1 or NFATp or NFATc was involved in gene induction at the Ets/NFAT sites of the mouse and human IL-5 proximal promoters. GATA-3 and GATA-4 had strong transactivational activity with the human IL-5 promoter and both cooperated well with Ets1 and AP-1. However, GATA-3 is the Th2 specific factor and therefore most likely to be involved in IL-5 expression.

Activation of the ERK MAP kinase pathway was sufficient to induce IL-5 activation in the absence of stimulation. The JNK pathway gave less gene induction and the p38 pathway gave very little activation. However, in stimulated cells, activation of each of the three MAP kinase pathways gave strong induction of IL-5 gene expression. These inductions were dependent on the AP-1, Ets/NFAT and GATA binding sites within the proximal promoter region of the mouse IL-5 gene. Taken together, these studies suggest that the proximal regions of both the human and mouse IL-5 promoters are critical for induction of the IL-5 gene. In addition, cooperative interactions between AP-1, Ets1 and GATA-3 are obligatorily required for IL-5 expression and may be part of a higher order transcriptional complex in activated T lymphocytes analogous to the enhanceosome of the IFN-β gene. Preliminary studies showed two sites of binding for HMGI(Y) in the proximal promoter and suggest that it may participate as an architectural transcription factor. The results support the proximal promoter region as the major inducible switch regulating IL-5 gene expression in T cells

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LIST OF ABBREVIATIONS

μCi	microCurie
μg	microgram
μl	microlitre
AP-1	activator protein-1
ATP	adenosine triphosphate
BSA	bovine serum albumin
bp	base pair
CsA	cyclosporin A
cAMP	dibutyryl cyclic adenosine monophosphate
cDNA	complementary DNA
CoA	coenzyme A
cpm	counts per minute
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
DMSO	dimethylsulphoxide
EDTA	elthylenediaminetetraacetic acid
EMSA	electrophoretic mobility shift assay
FCS	fetal calf serum
GM-CSF	granulocyte macrophage-colony stimulating factor
GR	glucocorticoid receptor
HEPES	N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)
HMG	high mobility group
hIL-	human interleukin-
IL	interleukin
INFβ	interferon β
kb	kilo base
kD	kilodalton
LB	Luria broth
mIL-	mouse interleukin-
M	molar
mg	milligram
ml	millilitre
mM	millimolar
mRNA	messenger ribonucleic acid
NFAT	nuclear factor of activated T-cells

ng	nanogram
nm	nanometre
NP-40	Nonidet P-40
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PKA	protein kinase A
PKC	protein kinase C
PMA	phorbol-12-myristate-13-acetate
PNK	polynucleotide kinase
rpm	revolution per minute
RNA	ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
SDS	sodium dodecyl sulfate
TAE	tris-acetate-EDTA
TBE	tris-borate-EDTA
$T_{10}E_1$	10 mMTris-hydrochoride, 1 mM EDTA. pH8.0
TCR	T-cell receptor
TNF	tumour necrosis factor
Tris	tris(hydroxymethyl) amino-methane
UTR	untranslated region

Chapter 1 General Introduction

1.1. Introduction

T lymphocytes coordinate the cellular immune response. Resting T lymphocytes are activated when their T cell receptor encounters foreign antigen presented in association with self-histocompatibility molecules on the surface of specialised antigen-presenting cells (Unanue and Allen, 1987). This specific activation via the T cell receptor in conjunction with other cell surface receptors leads to second messenger cytoplasmic activity which in turn ultimately affects transcription of particular sets of genes in the T cells (Weiss et al., 1986). The immunoregulatory effector molecules, known collectively as cytokines, are a major class of genes switched on by this signaling event.

Interleukin-5 (IL-5) is one of the key cytokines produced by activated T lymphocytes that are involved in the regulation of immune and inflammatory responses. IL-5 appears to play central role in the regulation of the eosinophilia and contributes to several human diseases, including asthma (Sanderson et al., 1988). In both atopic and non-atopic asthma, elevated IL-5 levels have been detected in peripheral blood and in the airways (Hamid et al., 1991; Walker et al., 1991; Robinson, et al., 1992). Understanding the regulation of this inducible cytokine may ultimately be valuable in treating certain pathological conditions in humans involving eosinophilia. Therefore, there is considerable interest in the identification of the transcriptional mechanisms regulating the expression of IL-5.

1.2. Molecular Biology of IL-5

1.2.1. Interleukin-5 gene and its structure.

The interleukin-5 gene is located on chromosome 11 in mouse and chromosome 5 in human. The exon/intron structure of the IL-5 genes in the mouse, human and rat is very similar having 4 exons and 3 introns. A similar gene structure arrangement is also found in other cytokine genes like IL-2, IL-4, GM-CSF and IFNγ. The 3 introns of the mouse IL-5 gene are 829, 1875 and 79 bp in length. The first 2 introns are significantly longer than those in the human gene which has introns of 208, 950 and 105 bp respectively. The relative positions of the introns within the coding regions are identical in the two genes and all introns begin with GT and end in AG. A potential TATA box is located 29 bp (human) and 30 bp (mouse) upstream from the start of transcription (Sanderson et al., 1988). The amino acid sequence of IL-5 is highly conserved between human and mouse. (78% homology). The IL-5 gene is part of a cytokine cluster on chromosome 11 in mouse and chromosome 5 in human with the IL-3, IL-4, IL-13 and GM-CSF genes (see below), suggesting these genes may be derived by duplication from a common ancestral gene (van Leeuwen et al., 1989). There is a significant homology between the IL-5 and GM-CSF genes from -53 to -39 in the proximal promoter region (Campbell et al., 1988; Mizuta et al., 1988). This element has been referred as CLE0 (conserved lymphokine element 0) and it is also present in the IL-4 and G-CSF genes (Miyatake et al., 1991). The highly conserved CLE0 element, the chromosomal clustering of the IL-5, IL-4 and GM-CSF genes and their inducible expression in T lymphocytes raises the possibility of coordinate regulatory mechanisms in their regulation.

1.2.2. Structure of the IL-5 protein

IL-5 is a homodimeric glycoprotein connected by two cysteine residues (Minamitake et al., 1990). The X-ray structure of IL-5 was first solved using the bacterially derived protein (Milburn et al., 1993) and was subsequently solved for recombinant protein derived by expression from baculovirus infection of Sf9 cells (Tavernier et al., 1995) and from Drosophila cells (Johanson et al., 1995). The structure reveals a novel two-domain structure, with each domain consisting of a four-α-helix bundle. The four-helix bundles in IL-5 are composed of three helices from one subunit, with the fourth being provided by the other subunit, producing a dimerization organization which is unique to date to IL-5 (Chaiken and Williams, 1996). An important feature of the IL-5 is the pair of Cys residues which are involved in the formation of intermolecular disulfide bridges. The disulfide bond assignment of recombinant IL-5 shows that the intermolecular disulfide bonds linking the two subunits occur in a head-to-tail fashion where the Cys44 residue from each chain forms a disulfide with the Cys86 residue from the other (Minamitake et al., 1990; Proudfoot et al., 1991). These Cys residues appear to be essential for IL-5 function, since their removal by site-directed mutagenesis abolished biological activity of IL-5 (McKenzie et al., 1991b).

Native IL-5 was isolated initially from mouse T-cell supernatants and was shown to be a glycoprotein which exists as a homodimer (McKenzie et al., 1987). The protein was shown to have an apparent molecular mass of 45 kDa, but the cloning of the cDNA sequence showed that the protein component is composed of two amino acid chains with a molecular mass of 13 kDa for each polypeptide monomer (Kinashi et al., 1986). Human IL-5 has a similar monomeric polypeptide which had 70% sequence similarity to the mouse protein (Azuma et al., 1986; Campbell et al., 1987). The expression of the cDNA in eukaryotic systems has shown that it is a disulfide-linked homodimer with apparent

molecular mass of 40 to 50 kDa with O-linked glycosylation at Thr4 and N-linked glycosylation at Asn29 (Minamitake et al., 1990), implying that about half of the IL-5 consists of carbohydrate. However, the carbohydrate component does not appear to be essential for IL-5 activity, since the recombinant expression of the cDNA encoding the human sequence in *E. coli* produces fully active protein, as does the expression in baculovirus-infected Sf9 cells in the presence of tunicamycin (Tavernier et al., 1989). The non-essentiality of glycosylation for biological activity is not unique to IL-5. GM-CSF is even more active as the unglycosylated protein than the glycosylated form (Burgess, 1988).

1.2.3. IL-5 receptor

The IL-5 receptor (IL-5R), a member of the Type 1 cytokine receptor superfamily, consists of an IL-5-specific subunit, the IL-5 receptor α chain (IL-5Rα), and a second subunit, IL-5 receptor β chain (IL-5Rβ) (Takaki et al., 1990; Tavernier et al., 1991; Takaki et al., 1991; Murata et al., 1992; Takaki et al., 1993). The IL-5Rα cDNA encodes a 420 amino acid protein consisting of a leader sequence, an extracellular domain, a transmembrane domain, and a cytoplasmic domain (Murata et al., 1992). Both mouse and human IL-5Rα also occur in a soluble form (Takaki et al., 1990, Murata et al., 1992).

The extracellular portion of IL-5Rα is composed of three fibronectin type-III-like modules (Takaki et al., 1990; Murata et al., 1992). The two membrane proximal modules contain the conserved superfamily structures; four conserved cysteines in the middle domain and the WSXWS motif (Bazan et al., 1990) in the membrane proximal domain. The cytoplasmic domain of IL-5Rα has no significant homology with signaling molecules such as kinases, phosphatases, nucleotide binding proteins, and the src

homology domain. It has regions rich in proline (Pro-Pro-X-Pro) following the transmembrane domain (Takaki et al., 1990) that are well conserved between IL-5Rα and the receptors for IL-3, GM-CSF, prolactin, and growth hormone.

The IL-5Rα subunit binds IL-5 ligand with low affinity in the mouse but with relatively high affinity in the human. The IL5Rβ subunit does not bind IL-5 alone, but associates with IL-5Rα to enhance the affinity for IL-5 binding (Tominaga et al., 1992). The IL-5Rβ also enhances the efficiency of IL-5 internalization, which explains the reduction in surface expression of high-affinity IL-5R following IL-5 stimulation. The IL-5Rβ is absolutely necessary for signal transduction (Mita et al., 1993). There is no significant association between IL-5Rβ and soluble IL-5Rα, which explains the ability of soluble IL-5Rα to act as an antagonist. The IL-5Rβ subunit is identical to the β-chains of the IL-3 and GM-CSF receptors and is known as the β-common chain (Lopez et al., 1992). Recent studies on the structure of the complete extracelluar domain of the β-common chain identify a novel interlocking dimer structure (Carr et al., 2001). The sharing of the β-chain among the receptors for the IL-3, IL-5, and GM-CSF genes suggests a common signaling mechanism among these cytokines and may explain the overlapping role of these cytokines in the development of granulocytes, as well as the cross-competition observed for these cytokines when binding to their receptors.

1.2.4. Transmembrane signaling by IL-5

Receptor-driven signaling processes typically originate from the juxtamembranous region of the receptor. The molecular mechanism by which IL-5 binding to its receptor elicits diverse responses in subsets of leukocytes remains an enigma. Accumulating evidence suggests that phosphatidyl inositol turnover, calcium mobilization and

activation of protein kinase A are not essential for IL-5-induced differentiation signals (Yamaguchi et al., 1989). Although tyrosine phosphorylation is usually one of the earliest biochemical events in the signal transduction cascade induced by growth factors whose receptors possess endogenous enzymatic activity (Ullrich and Schlessinger et al., 1990), the cytoplasmic domains of IL-5Rα and the βc subunit, like a number of other cytokine receptors, have no homology with tyrosine kinases. Nevertheless, IL-5 does induce rapid tyrosine phosphorylation of cellular proteins (Morla et al., 1988; Murata et al., 1990), and enhances the transcription of nuclear proto-oncogenes (Conscience et al., 1986). The suppression of IL-5-induced proliferation with a tyrosine kinase inhibitor (Sato et al., 1994) indicates that activation of tyrosine kinases is indispensable in IL-5 signal transduction.

One of the earliest events that occur after IL-5 binds to its receptor is the rapid tyrosine phosphorylation of βc and several cellular substrates including Shc and Vav. The tyrosine phosphorylated Shc can activate the Ras pathway by binding to an adapter protein called Grb2 through its SH2 domain (Pelicci et al., 1992; Egan et al., 1993). Grb2 also binds through its SH3 domain to a proline-rich motif of Sos, a nucleotide exchange protein. Sos then activates Ras, which is a GTP binding protein that functions downstream of tyrosine kinases in the growth signaling pathway (Smith et al., 1986; Satoh et al., 1991; Duronio et al., 1992). Downstream of Ras is a serine/threonine kinase called Raf which activates the unique bifunctional kinase MEK that can phosphorylate both tyrosine and serine/threonine. The known substrate for MEK is the serine/threonine kinase MAP kinase. Once activated, MAP kinase triggers nuclear transcription factors that lead to the induction of several gene products including proto-oncogenes. Activation of the Ras cascade is dependent on both the cytoplasmic region of the IL-5Rα and the region between Leu626 and Ser763 of βc (Gorman et al., 1992). The induction of

tyrosine phosphorylation and nuclear proto-oncogene expression is critical for cytokine signal transduction (Conscience et al., 1986). Expression of c-Fos, c-Jun, and c-Myc proto-oncogene mRNA is rapidly induced upon stimulation with IL-5 (Takaki et al., 1994). This proto-oncogene expression is not induced in cells transfected with IL-5Rα mutants that lack either the proline-rich motif or a sequence just downstream of the proline-rich region in the cytoplasmic domain (Takaki et al., 1994).

Janus kinases (JAK) are a family of cytoplasmic protein tyrosine kinases including JAK-1, JAK-2, JAK-3, and TYK-2 and recent studies have revealed that the JAK family of kinases couples ligand binding to cytokine receptors with tyrosine phosphorylation of cellular proteins (Ihle, 1995). JAK-2 is rapidly tyrosine phosphorylated in response to IL-5 (Kouro et al., 1996). A significant but lesser extent of tyrosine phosphorylation of JAK-1 is also observed in response to IL-5 (Kouro et al., 1996). The membrane-proximal proline-rich sequence of the cytoplasmic domain of IL-5 Rα, which is also conserved in the α chains of IL-3Rα and GM-CSFRα, is essential for tyrosine phosphorylation of JAK-2 (Cornelis et al., 1995). Treatment of human peripheral blood monocytes or basophils with IL-3, IL-5, or GM-CSF activates DNA binding proteins which are tyrosine-phosphorylated (Larner et al., 1993). An IL-3 and GM-CSF responsive STAT protein has been identified as STAT-5 (Mui et al., 1995), which is one of the substrates for JAK-2 kinase. IL-5 also induces STAT-5 activity (Kouro et al., 1996; Mui et al., 1995; Ogata et al., 1997), which is mediated by both JAK-2 and to a lesser extent JAK-1. Activation of JAK-2 and STAT-5 has been shown in eosinophils in response to IL-5 (Ogata et al., 1998; Pazdrak et al., 1995). The activation of STAT-5 in response to IL-3, IL-5 and GM-CSF suggests a common signaling pathway through the IL-3R, IL-5R, and GM-CSFR, all of which share βc. These studies suggest different JAK-STAT signaling pathways for B cells and eosinophils.

1.3. Biological function of IL-5

1.3.1. Eosinophils

Eosinophilia involves four different processes: differentiation of progenitor cells and proliferation of eosinophils in bone marrow; interactions between eosinophils and endothelial cells that involved rolling, adhesion, and migration of eosinophils; chemoattraction directing eosinophils to a specific location; and activation and destruction of eosinophils (Rothenberg, 1998). Eosinophils are produced in bone marrow from pluripotent stem cells. The latter cells differentiate first into hybrid precursors with properties of basophils and eosinophils and then into a separate eosinophil lineage (Boyce et al., 1995). There is strong evidence that an excess of eosinophils and their products are involved in the pathogenesis of allergic disease such as bronchial asthma (Hoidal, 1990). Eosinophils have been associated with neoplasia in various types of tumour-associated eosinophilia (Lowe et al., 1981) and are believed to be involved in host defence against helminth infections (Hagan et al., 1985).

The most obvious feature of asthma is bronchoconstriction associated with an acute attack. However, the major underlying cause of an asthma attack is chronic pulmonary inflammation characterised by decreased mucociliary clearance, edema, epithelial damage, and broncho-alveolar eosinophilia (Mahanty and Nutman, 1993). There is increasing evidence that eosinophils are involved in the pathophysiology of asthma, as shown by the ability of eosinophil products such as major basic protein, eosinophilic cationic protein, and eosinophil peroxidase to cause damage to lung epithelium both *in vitro* and *in vivo* (Kay et al., 1995). Three cytokines, IL-3, IL-5 and GM-CSF, are particularly important in regulating the development of eosinophils.

1.3.2. Regulation of eosinophils by Th2 lymphoctes and mast cells

Mast cells participate in the initial events after exposure to allergen, but their importance in orchestrating eosinophilia is uncertain. After IgE-triggered activation, mast cells may promote inflammation of the airways with eosinophils by producing proinflammatory mediators (e.g., IL-1 and TNFα) and eosinophil-directed cytokines (e.g., IL-4 and IL-5). These substances, in turn, induce chemokines that attract eosinophils. However, mast cells do not appear to be required in some animal models of allergic disease. In allergen-sensitized mice with a deficiency of mast cells and allergen-sensitized mice with a targeted deletion of the gene for IgE, recruitment of eosinophils into the lungs is not impaired after allergen challenge (Mehlhop et al., 1997; Hamelmann et al., 1997; Brusselle et al., 1994). In contrast, helper T lymphocytes are essential for the late-phase allergic response, because they produce three cytokines that promote allergic responses: IL-4 and IL-13, both of which regulate IgE and VCAM-1 production, and IL-5. The helper cells that orchestrate this type of response are Th2 cells. In contrast, Th1 cells produce IFNγ and TNFβ (Mosmann and Coffman, 1989; Robinson et al., 1992). Genetic factors and the conditions of antigen exposure determine the relative contributions of mast cells and T cells in the regulation of eosinophils (Galli, 1997).

1.3.3. IL-5 and Eosinophils

Among the cytokines which can support eosinophilopoiesis, IL-5 is unique in its ability to specifically promote the terminal differentiation and maturation of eosinophil/basophil lineage-committed progenitors (Clutterbuck et al., 1989). In addition, IL-5 prolongs eosinophil survival by delaying apoptotic cell death (Yamaguchi et al., 1988). IL-5 also primes and stimulates eosinophil chemotactic activity (Sehmi et al., 1992), and enhances eosinophil effector function (Yamaguchi et al., 1988). A pivotal role

for IL-5 in chronic allergic inflammation has been confirmed by the capacity of neutralizing anti-IL-5 mAb to inhibit antigen- or virus-induced airway hyperresponsiveness and eosinophil infiltration in the airways of mice, guinea pigs and primates (Nagai et al., 1993; Kung et al., 1995; Chand et al., 1992; Mauser et al., 1995; Egan et al., 1995). These findings suggest that IL-5 antagonists or drugs blocking IL-5 production may provide a new therapeutic approach to these allergic disases. Leckie et al., 2000

Of the three cytokines (IL-3, GM-CSF and IL-5), IL-5 is the most specific for the eosinophil lineage and is responsible for selective differentiation of eosinophils (Sanderson, 1992). In asthma patients, high levels of IL-5 mRNA in the bronchial mucosa is detected (Hamid et al., 1991). IL-5 protein is also detectable in the bronchoalveolar lavage (BAL) fluid from symptomatic asthma patients (Ohnishi et al., 1993). IL-5 also stimulates the release of eosinophils from bone marrow into the peripheral circulation (Collins et al., 1995). The critical role of IL-5 in the production of eosinophils is best demonstrated by genetic manipulation in mice. Overproduction of IL-5 in transgenic mice results in profound eosinophilia (Sanderson, 1992; Takatsu et al., 1994) and deletion of the IL-5 gene causes a marked reduction of eosinophils in blood and lungs after an allergen challenge (Foster et al., 1996) or after helminth parasite infection (Kopf et al., 1996) In allergic lung inflammation tissue, eosinophilia was shown to depend on the cooperation of IL-5 with IL-4 and IL-13 (Grunig et al., 1998). IL-4 and IL-13 promote eosinophil extravasation, at least in part, by upregulating the expression on endothelial cells of vascular cell adhesion molecule (VCAM)-1, a major adhesion molecule for eosinophils, which express the receptor VLA-4 (very late antigen-4) on their membranes (Schleimer et al., 1992). IL-4 and IL-13 stimulate the production of eotaxin by several cell types (Rothenberg et al., 1995). In conjunction with IL-5, eotaxin is crucial for eosinophil chemotaxis (Collins et al., 1995; Mould et al., 1997) and

its receptor, CCR3, is considered as a potential target for anti-allergic therapies. The overproduction of one or more of the these cytokines occurs in humans with eosinophilia. Diseases involving eosinophilia without increases in other blood-cell lineages are usually accompanied by an overproduction of IL-5 (Sanderson, 1992). The mechanisms of cytokine overproduction may involve a response of T-helper lymphocytes of the Th2 type in patients with allergic conditions or parasitic diseases (Mosmann and Coffman, 1989; Robinson et al., 1992), the malignant expression of T-cell clones that produce IL-5 in some patients with lymphoma (Cogan et al., 1994) or the activation of gene transcription due to a chromosomal translocation in some patients with leukemia (Grimaldi and Meeker, 1989; Liu et al., 1993).

1.3.4. IL-5 and parasite infections

The primary function of eosinophils is believed to be host defence against infection by relatively large organisms such as parasitic helminths. This is based on the finding that eosinophils degranulate on to and kill helminths *in vitro* in the presence of antibody and /or complement; they move from the blood and aggregate in the locality of helminths *in vitro*; large numbers of eosinophils are often seen in close association with both intact and damaged helminths *in vivo*; and they clearly degranulate in the vicinity of surface of helminths *in vivo* (Butterworth, 1984).

A variety of studies have been carried out in which monoclonal antibodies (mAbs) that neutralize IL-5 have been administered to mice. This treatment greatly reduced the development of eosinophilia upon infection with parasitic helminths, but had little effect onthe survival or reproduction of a number of nematodes and trematodes (Coffman et al., 1989; Sher et al., 1990; Betts and Else, 1999; Rotman et al., 1996). However, in a

minority of studies with other parasite species, anti-IL-5 antibody treatment exacerbated the infection. The survival and distribution of parasites in certain tissues was increased in primary infections of mice with the rat parasites *Strongyloides venezuelensis* or *Angiostrongylus cantonensis* (Korenaga et al., 1994; Sasaki et al.,1993) in which eosinophils were ablated. Anti-IL-5 mAb treatment compromised both the killing, by eosinophils, of *Onchocerca volvulus* infective larvae implanted in diffusion chambers of vaccinated mice and the clearance of microfilariae of *Onchocerca linealis* from immunized mice (Folkard et al., 1996). Similarly, killing of third-stage larvae of *Strongyloides stercoralis*, the parasite that infects humans, within diffusion chambers in immunized mice was ablated by IL-5 mAb treatment (Rotman et al., 1996).

Transgenic mice constitutively overexpressing IL-5 display constitutive high blood and tissue eosinophilia (Dent et al., 1990; Tominaga et al., 1991). These mice were infected experimentally with parasites and studies showed that parasite burdens were dramatically decreased (Yoshida et al., 1996; Dent et al., 1999; Shin et al., 1997), indicating an IL-5-dependent host-protective effect. Many of the *Nippostrongylus brasiliensis* worms that did establish in the intestine of the transgenic mice failed to thrive and produce eggs (Dent et al., 1999). However, worm burdens in secondary *Nippostrongylus brasiliensis* infections of normal and IL-5 transgenic mice were similar, indicating no essential role for IL-5 in immunological memory in this infection (Dent et al., 1999). Worms in the IL-5 transgenic mice were killed more rapidly which correlated with greatly intensified eosinophil infiltration into the cerebrospinal fluid of transgenic mice and increased parasite antigen-specific serum IgG1 and IgA (Daly et al., 1999). IgA is reported to be the most effective stimulator of degranulation of human eosinophils (Fujisawa et al., 1990).

Recently, studies with IL-5-deficient (Kopf et al., 1996) and IL-5Rα-deficient (Yoshida et al., 1996) mice provided further evidence that IL-5 plays an important role in host defence against some parasites. The IL-5Rα is exclusive to the IL-5 receptor, and is expressed in eosinophils and $CD5^+$ B-1 cells which are responsive to IL-5. IL-5- and IL-5Rα-deficient mice harbour very small populations of normal eosinophils and fail to develop an eosinophilopoietic response when infected with any of the helminths tested (Ovington et al., 1997). IL-5Rα-deficient mice have reduced levels of serum IgM and IgG3 and mucosal secretory IgA. The effect of infection of these mice with a large variety of parasites such as *Mesocestoides corti* (Kopf et al., 1996), *Hymenolepis diminuta* and *Fasciola hepatica* (Ovington et al., 1997), *Trichinella spiralis* (Vallance et al., 1999), *Strongyloides ratti* (Ovington et al., 1998), *Toxocara canis* (Takamoto et al., 1997), *Angiostrongylus cantonensis* (Yoshida et al., 1996; Sugaya et al., 1997) has been determined. Worm burdens and distributions in primary infections IL-5-deficient mice with *Mesocestoides corti* were similar to wild-type mice although reduced pathology in *Toxocara canis* infections was observed (Kopf et al., 1996; Takamoto et al., 1997). In the infections of IL-5-deficient mice with *Trichinella spiralis*, reduced smooth muscle hypercontractility and a delay in expulsion of intestinal adults were also observed although worm burdens were similar (Vallance et al., 1999). IL-5-deficient mice infected with *Strongyloides ratti* showed reduced host defence against this intestinal nematode (Ovington et al., 1998). These studies in mice give insight into *in vivo* functions of IL-5 and eosinophils.

1.3.5. IL-5 and graft rejection

Eosinophil infiltration has been reported to be a specific indicator of adverse prognosis in renal (Kormendi and Amend, 1988) liver (Foster et al., 1989) and lung

(Kondo et al., 1991) transplantation. IL-5 expression in biopsies from human liver allografts correlates with histopathological evidence of acute rejection (Martinez et al., 1992). Both IL-5 transgenic mice and neutralizing IL-5 antibodies have been used to investigate the role of eosinophils in transplant rejection. IL-5 antibody treatment fully delayed allogeneic heart graft rejection and facilitated tolerance induction with anti-T-cell monoclonal antibody therapy. Despite the disappearance of the heavy eosinophilic infiltrates observed in untreated recipients, IL-5 inhibition did not modify the rejection of pig pancreatic islets in mice, suggesting that eosinophils are essentially innocent bystanders in this model (Simeonovic et al., 1997). IL-5 neutralization only slightly affected the survival of fully mismatched heart allografts in CD8+ T-cell-depleted mice recipients although the eosinophilic infiltration of the grafts was strictly IL-5 dependent (Braun et al., 2000). Studies with mice in which skin-graft rejection is triggered by a single MHC class II incompatibility showed that the CD4+ T-cell response in this model is Th-2 biased and associated with massive eosinophilic infiltrates, which depend on IL-5 production (Le Moine et al., 1999a). Although IL-5 neutralization significantly prolonged graft survival it was unable to prevent rejection in wild-type animals, as CD4+ T cells use two pathways to induce graft damage in this model (Le Moine et al., 1999a). The first pathway depends on FasL-Fas signaling whereas the second pathway involves IL-5 and eosinophils. Indeed, when skin transplantation was performed using FasL-deficient mice as recepients or Fas-deficient mice as donors, IL-5 neutralization completely prevented graft rejection (Le Moine et al., 1999a). This suggests that the role of IL-5-eosinophil pathway in transplant rejection becomes crucial when the cytotoxic CD8+ T-cell pathway is not operative and the FasL-Fas pathway of CD4+ T-cell-mediated rejection is blocked.

IL-5 neutralization, by injection of an anti-IL-5 antibody or the use of IL-5-deficient mice as recipients, established a crucial role for eosinophils in the induction of

fibrosis. However, IL-5-deficient mice develop transplant arteriosclerosis, indicating that the vascular lesions did not depend on eosinophils (Le Moine et al., 1999a). IL-4 appeared to be a crucial mediator of vasculopathy (Le Moine et al., 1999b).

1.3.6. Role of IL-5 in B-cell development

IL-5 is a synonym of T-cell replacing factor, B-cell growth factor II, IgA enhancing factor, eosinophil differentiation factor, and eosinophil colony-stimulating factor (Kinashi et al., 1986; Takatsu et al., 1994). IL-5 induces DNA synthesis in mouse chronic leukemic B cells and dextran-sulfate-stimulated splenic B cells (Hitoshi et al, 1990). In mice expressing the IL-5 transgene, the B-1 cell population is markedly increased with concomitant hypergammaglobulinemia and autoantibody production (Tominaga et al., 1991). IL-5- and IL-5Rα-deficient mice show decreased numbers of B-1 cells and peripheral eosinophils concomitant with low serum concentrations of IgM and IgG3 (Yoshida et al., 1996; Kopf et al., 1996). IL-5 acts on appropriately activated B cells to induce IgG1 and IgA production (Takatsu et al., 1994). Thus evidence has accumulated in mouse systems for both an IL-5-sensitive developmental pathway for B-1 cells and a role for IL-5 in the terminal differentiation of mature B-1 cells and appropriately activated B-2 cells (Takatsu et al., 1994). In addition, a role for IL-5 in promoting C_H gene recombination has been demonstrated by synergy with IL-4 which induces recombination in mouse B cells activated with anti-IgD dextran (Mandler et al., 1993). IL-5 is therefore a potent cytokine to induce terminal differentiation of activated mouse B cells and a priming factor to induce accessibility to other cytokines.

In contrast to mouse B cells, evidence of a role for IL-5 in human B-cell growth and differentiation has been controversial (Huston et al., 1996; Clutterbuck et al., 1987).

No effects on proliferation and Ig production were found when IL-5 was tested for functional activity on purified human B cells from peripheral blood or spleen using anti-IgM antibodies (Clutterbuck et al., 1987). Conversely, other studies subsequently reported the potential of human B cells to respond to IL-5. As with mice, IL-5 responsiveness of human B cells depends on the activation signal used. Detection of message for IL-5Rα in resting B cells upon the stimulation of B cells with the novel bacteria B-cell mitogen *Moraxella catarrhalis* and Ig synthesis of B cells stimulated with mitogen and IL-5 have provided direct evidence that human B cells are able to respond to IL-5 (Huston et al., 1996)

1.4. Expression of the IL-5 gene

1.4.1. Gene clustering and coordinated expression of cytokine genes

There is now well-documented evidence that eosinophilia is primarily regulated by the production of IL-5, but also by the expression of IL-4 and by the recently described IL-13 (Sanderson et al., 1992, Minty et al., 1993, Zurawski et al., 1994). A number of other cytokines, including IL-3, GM-CSF, IL-9, and IL-10 influence the immediate inflammatory responses elicited during allergic reactions. It is noted that a number of these genes, including IL-3, IL-4, IL-5, IL-13, and GM-CSF, are located in a cluster on mouse chromosome 11 and human chromosome 5 (van Leeuwen et al., 1989; Mckenzie et al., 1993). With the exception of IL-13 (which has five exons), the genes within this cluster share a common intron/exon structure consisting of four exons and three introns (Mckenzie et al., 1993). The human IL-13 gene is upstream and probably within 20 kb of the IL-4 gene (Morgan et al., 1992). Physical linkage has also been demonstrated between the IL-4 gene and IL-5 gene in human and mouse, with approximately 310 kb

separating them in human (Chandrasekharappa et al., 1990) and approximately 110 to 180 kb in mouse (Lee et al., 1989). The human IL-3 and GM-CSF genes are extremely close linked and map in the region of 600 to 1000 kb from IL-5 (Le Beau et al., 1989). Recent studies using cross-species sequence comparisons followed by characterization of conserved regions in yeast artificial chromosome transgenic mice have identified a coordinate regulator of IL-4, IL-13 and IL-5 expression (Loots et al., 2000). The structural relationship and clustering of the IL-13, IL-4, IL-5, IL-3, and GM-CSF genes suggest that they have evolved through ancestral gene duplication. Although these cytokines may show coordinate expression, it is evident that they are also individually regulated (Naora and Young, 1995).

1.4.2. Role of the cytokines produced by Th1 and Th2 cells

Native CD4+ helper T cells recognize specific major histocompatibility complex (MHC)-peptide combinations on antigen-presenting cells (APCs) via interactions with the T-cell receptor (TCR), providing the first signal required for activation. The second, costimulatory signal, is provided by accessory molecules expressed on APCs, such as the B7 family of proteins. B7 proteins are the ligands for the CD28 and CTLA-4 costimulatory molecules that are expressed on T cells. The combination of these two signals induces IL-2 synthesis and secretion, IL-2 receptor expression, clonal expansion and differentiation of precursor CD4+ T cells into effector T helper (Th) cells (Rincon and Flavell, 1997).

Activated CD4+ T cells are classified into two distinct subsets (Th1 and Th2) based on their biological functions, which in turn, depend on the cytokines they express. Th1 cells produce inflammatory cytokines, such as TNF-β and IFN-γ, and enhance cellular

immunity. Th2 cells produce a different group of cytokines- IL-4, IL-5, IL-6, IL-10, and IL-13, and help B cells secrete antibodies. The differentiation of native T cells into the Th1 or Th2 phenotype has important biological implications in terms of susceptibility or resistance to a particular disease.

The cytokines themselves play the most critical role in T helper cell polarization. The two critical cytokines that control Th1 and Th2 differentiation are IL-12 and IL-4, respectively. These two cytokines enhance the generation of their own Th subset and simulaneously inhibit the generation of the opposing subset (Macatonia et al., 1993; Manetti et al., 1993; Powrie and Coffman, 1993; Trinchieri, 1993). These events are likely to occur at a precursor stage (Thp) because, once established, Th1 and Th2 cells are stable (Scott, 1991; Murphy et al., 1996). The requirement for IL-12 and IL-4 has been demonstrated unequivocally by the phenotype of mice that lack these cytokines, their receptors, or the effector molecules downstream of receptor signaling. IL-12, secreted by APC, activates the STAT4 signaling pathway, and mice lacking IL-12 or STAT4 do not have Th1 cells (Kaplan et al., 1996; Magram et al., 1996; Thierfelder et al., 1996). Two other cytokines that influence Th1 development are IL-18, whose receptor is related to the IL-1 receptor family (Okamura et al., 1995), and IFN-γ (Scott, 1991; Meraz et al., 1996). IFN-γ activates the STAT1 pathway (Meraz et al., 1996). Mice lacking IL-18 or STAT1 have defective *in vivo* Th1 responses (Meraz et al., 1996; Takeda et al., 1998). In contrast, mice that lack IL-4, IL-4 receptor or STAT6, the downstream signaling molecule for the IL-4 receptor, fail to develop Th2 cells in response to most stimuli (Kuhn et al., 1991; Kopf et al., 1993; Shimoda et al., 1996; Takeda et al., 1996; Noben-Trauth et al., 1997). The biological function of IL-13 partially overlaps with IL-4 because, in some instances, IL-13 drives Th2 development and IgE synthesis in an IL-4-independent fashion (Minty et al., 1993; Punnonen et al., 1993 Emson et al., 1998;

McKenzie et al., 1998). IL-13 is especially important in the asthmatic response (Wills-Karp et al., 1998; Wills-Karp, 2001)

The Th1/Th2 differentiation step requires new protein synthesis and gene expression. An understanding of the transcriptional mechanisms that are involved in the differentiation and /or activation of Th1 and Th2 cells might provide potential targets for therapeutic intervention.

1.4.3. Signaling pathways involved in IL-5 regulation

In order to establish whether IL-5 is regulated by mechanisms shared with other coordinately expressed cytokines in Th2 cells, the effect of variety of activators and inhibitors of known signaling pathways on the expression of IL-5 and five other cytokines was studied in the Th2 clone D10 (Naora et al., 1994a; Naora and Young, 1995). Using northern blot analysis it was shown that, whereas none of the six cytokine mRNAs could be detected in unstimulated D10 cells, the lectin Con A was effective in inducing all of the cytokine genes. This agent is believed to stimulate a number of glycosylated cell surface molecules including the TCR-CD3 complex which triggers activation of two parallel second messenger pathways involving PKC activation and Ca^{2+} mobilization. The Ca^{2+} signal appears to be largely mediated via a CsA-sensitive pathway in D10 cells and this pathway was shown to be essential for TCR-CD3-induced expression of the IL-3, IL-4, and GM-CSF genes. The Ca^{2+} signal was optimally induced by the Ca^{2+} ionophore A23187 which produces sustained elevation of intracellular Ca^{2+} levels. In contrast, the TCR-CD3-stimulated expression of the IL-5, IL-6, and IL-10 genes was relatively resistant to CsA. Furthermore, these three genes were induced by PMA stimulation alone, suggesting that the PKC signaling pathway may independently

regulate these genes. The IL-5, IL-6, and IL-10 gene were also selectively induced by stimulation of the IL-1 receptor and by forskolin. Studies by other workers with a subline of D10 (Munoz et al., 1990) have indicated that stimulation of the IL-1 receptor induces PKC activation and increased intracellular cAMP flux. Since forskolin activates adenylate cyclase it would appear that the IL-5, IL-6, and IL-10 genes can be induced by PKC- and cAMP-dependent pathways in D10 cells.

1.5. Transcriptional regulation of cytokine genes

1.5.1. Basal transcriptional machinery

In the eukaryotic cell, messenger RNA (mRNA) synthesis of most genes is controlled by the enzyme RNA polymerase II. Although the eukaryotic RNA polymerase II is able to catalyze mRNA synthesis, it is not sufficient by itself to initiate transcription. Instead, a host of general transcription factors (GTFs), including TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH, work in a concert with RNA polymerase II to bring about promoter recognition and accurate transcriptional initiation (Zawel et al., 1995). The complete set of GTFs is usually sufficient to direct a basal level of transcription *in vitro* from strong promoters. Based on the TATA box (-TATA-DNA sequence), eukaryotic gene promoters are divided into two groups: 1) TATA-containing promoters that have the -TATA- sequence upstream of the transcriptional induction site and possess strong promoter activity; and 2) TATA-less promoters that do not have a -TATA- sequence upstream of the transcriptional induction site and express relatively weak promoter activity . So far, all cytokine gene promoters are TATA-containing promoters.

Transcription from a cytokine gene promoter is tightly controlled by the combined actions of positive and negative sequence-specific DNA-binding nuclear factors. Activators are transcription factors that can enhance promoter activity. Chromatin-associated proteins such as HMG proteins and histone proteins also play an important role in transcriptional regulation. For transcription to occur, a complex array of signals must be integrated at the gene promoter to initiate RNA production.

1.5.2. Regulation of the basal transcriptional machinery

One paradigm that has been established as a result of *in vitro* biochemical studies with RNA polymerase II is the importance of protein-protein interactions between activator proteins and components of the basal transcriptional machinery. Site-specific transcriptional activators or repressors, which recognize and bind to specfic DNA sequences in the genomic DNA, target distinct components of the basal transcriptional machinery. For example, the nuclear factor Sp1 can specifically interact with a subunit of the general transcription factor TFIID (Pugh and Tjian, 1991; Pugh and Tjian, 1990). TFIID is a large complex in the basal transcription machinery that contains at least nine subunits, including the TATA binding protein (TBP) and another eight or more transcription-associated factors(TAFs). The Sp1-specific subunit of TFIID has a molecular mass of 110 kDa and interacts with the glutamine-rich activation domain of the Sp1 protein (Tanese et al., 1991). Therefore, when Sp1 binds to a promoter in which the 110 kDa subunit is required for the activity of TFIID (Tanese et al., 1991), Sp-1 may facilitate assembly of the basal transcription machinery and enhance promoter activity. If the 110 kDa subunit is not necessary for promoter activity, Sp1 may not regulate the activity of the promoter even if it can bind to the promoter DNA. This specific interaction

provides a model of how a specific DNA-binding nuclear factor regulates assembly and function of the basal transcription machinery.

In some cases, the site-specific nuclear factor cannot physically contact a component of the basal transcriptional machinery, but may indirectly interact with the basal transcriptional machinery through an intermediate nuclear factor known as a "cofactor". For example, CREB (CRE binding protein) is a nuclear factor that recognizes the cyclic AMP response element (CRE) in many gene promoters (Foulkes and Sassone-Corsi, 1996). However, in the absence of a nuclear factor such as CBP (CREB binding protein), the DNA-bound CREB cannot exert its cis-acting function on the promoter because it can not directly interact with the basal transcriptional machinery (Chrivia et al., 1993; Kwok et al., 1994). CBP is a non-DNA-binding nuclear factor that is able to bind to CREB through protein-protein interactions. However, CBP can also interact physically and functionally with the general transcription factors TFIIB and TBP (Janknecht and Hunter, 1996). In this case, DNA-bound CREB influences assembly of the transcription initiation complex through the CBP protein; thus, CBP acts as a cofactor that mediates signals between specific nuclear factors and the basal transcriptional machinery.

1.5.3. Chromatin remodeling in cytokine gene expression

1.5.3.1. Role of chromatin structure

Genomic DNA is packed into nucleosomes, which then form higher order chromation structures. Chromatin remodeling is accompanied by changes in nucleosomal positioning which is ATP dependent. It has been suggested that cytokine gene loci, including IL-2, IL-4, IL-12, IL-13, IFNγ and GM-CSF, undergo changes in chromatin structure that allow access to gene-specific transcription factors, likely mediated in part

through acetylation of histones. Distal control regions such as enhancers and LCRs (locus control regions) have been shown to regulate accessibility to gene loci by regulating chromatin structure (Ernst and Smale, 1995). The location of regions that control these changes have been identified in cytokine genes using both assessment of CpG methylation status and DNase I hypersensitivity assays since transcriptionally active chromatin is hypomethylated and is more accessible to nucleases (Agarwal and Rao, 1998; Kadonaga, 1998; Agarwal et al., 1999). The most complete information on the role of chromatin remodeling in the regulation of cytokine genes is available for IL-4 and IFN-γ.

1.5.3.2. Evidence for chromatin remodeling in cytokine genes

Several observations suggested that the IL-4 gene was regulated at the level of chromatin structure. First, *in vitro* analyses had indicated that only 157 bp of the proximal IL-4 promoter was required for tissue-specific expression in Th2 cells. *In vivo*, the proximal 800 bp of the promoter was required to confer significant Th2-selective expression of an IL-4 promoter-reporter transgene (Wenner et al., 1997) However, not even 3 kb of sequence upstream the promoter were sufficient to achieve expression equivalent to the endogenous IL-4 gene, suggesting the presence of additional elements for optimal expression (Todd et al., 1993). Second, differentiated effector Th2 cells produce IL-4 more rapidly and at higher levels than naïve Thp cells, implying that the IL-4 regulatory regions are more accessible in effector, but not in naïve T cells (Reiner and Seder, 1999).

In both mouse and human chromosomes, the Th2 cytokine genes IL-4, IL-5 and IL-13 are clustered together within 150 kb, consistent with the notion that these genes compose a single chromosomal locus that may be controlled by long-range modulation of

chromatin structure. Evidence supporting this model was obtained using both methylation and DNase I hypersensitivity assays was obtained by identifying regions in the IL-4/IL13 locus that responded to stimulation through the TCR. Differentiated Th2 cells and established Th2 clones had an accessible chromatin structure as evidenced by the presence of five clusters of hypersensitive (HS) sites over the IL-4 locus. Naïve T helper cells, in contrast, like Th1 cells, possessed only one of the five HS sites. After 48 hours of antigen activation, naïve Thp cells rapidly acquired the chromatin phenotype of differentiated Th2 cells, implicating these regions in gene regulation (Agarwal and Rao, 1998). The Th2-specific transcription factor GATA-3 produced the same changes when transduced into differentiated Th1 cells or STAT6-deficient cells, indicating that GATA-3 acts to remodel this locus downstream of STAT6 (Ouyang et al., 2000). Factors like GATA-3 and STAT6 may directly remodel chromatin structure to allow TCR-induced factors like c-Maf and NFAT to access their specific binding sites in the IL-4 locus and promote rapid transcription of IL-4 (Agarwal and Rao, 1998). NFAT proteins may also directly alter chromatin configuration. Mice that lack both NFATp and NFATc largely overproduce Th2 cytokines and display a highly allergic phenotype (Ranger et al., 1998) ,suggesting that NFAT proteins may help regulate the balance between the active/inactive state of the IL-4/IL-5/IL-13 locus during the initiation of Th2 differentiation.

Although chromatin remodeling may also be important for the IFN-γ gene, less is known about this locus than the IL-4 locus. Tissue (Th1)-specific DNase I HS regions have been identified in both the first and third introns of the IFNγ gene (Young et al., 1994; Agarwal and Rao, 1998), and these assays also demonstrated alteration of the IFN-γ locus in native Th1 cells compared with Th2 cells (Agarwal and Rao, 1998). The methylation patterns of the IFNγ locus have also been analyzed during T-cell differentiation. IFN-γ-producer cells like Th1 and CD8+ T cells display hypomethylation

that appears to be a stable, long term inheritable trait (Young et al., 1994; Fitzpatrick et al., 1999), whereas the same locus is methylated in Th2 cells. The assembly of Th1-specific transcription factors may remodel the chromatin in a configuration that facilitates access to other factors required to transcribe the IFN-γ gene.

1.5.3.3. Role of the HMGI(Y) in cytokine gene expression

The mammalian HMG I(Y) family of proteins consists of three members: HMG I, HMG Y and HMG I-C. HMG I and HMG Y are encoded by the same gene and are generated through alternative RNA splicing, whereas a separated gene encodes HMG I-C (Bustin and Reeves, 1996). The HMG I proteins bind specifically to the minor groove of AT-rich regions of DNA via three short basic repeats containing the core motif GRGRP or PRGRP (Huth et al., 1997). The HMG I(Y) proteins can affect gene expression in different ways, including alteration of DNA conformation through DNA bending and distortion, increase of supercoiling of plasmid DNA (Nissen and Reeves, 1995), binding to entry/exit regions of nucleosomes (Reeves and Nissen, 1993), change of rotational setting of DNA on nucleosomes (Reeves and Nissen, 1993), enhancement or inhibition of transcription factor binding (Bustin and Reeves, 1996), binding to transcription factor consensus sites on gene promoters (Bustin and Reeves, 1996), or stabilization of enhancesome complexes on gene promoters (Yie et al., 1999).

There is increasing evidence that HMG I(Y) plays an important role in cytokine gene transcription. Many of the inducible cytokine genes contain a number of closely spaced HMG I(Y) binding sites overlapping or adjacent to other transcription factor binding sites in their promoter or enhancer regions. The multiple abilities of HMG I(Y) to alter DNA structure, modulate transcription factor binding and also to bind to nucleosomes may contribute to its influence on these gene promoters/enhancers. The

IFN-β promoter was the first cytokine gene promoter where HMG I(Y) was shown to be involved in transcription. IFN-β is induced by virus infection in mammalian cells, and the transcription factor complexes involved have been studied in detail. HMG I(Y) appears to have an important role in the activation of IFN-β transcription by facilitating the assembly of an enhanceosome at several organizational levels. HMG I(Y) can bind to four sites on the IFN-β promoter and promotes the binding of NF-kB and ATF/c-Jun complexes to the PRDII and PRDIV sites, respectively (Du et al., 1993; Thanos and Maniatis, 1992, 1995). The relative arrangement of the transcription factor and HMG I(Y) binding sites appears to be critical for the enhancement of factor binding by HMGI(Y). The specific directional binding as well as the molecular cooperativity of the two HMG I(Y) molecules bound to the promoter are critical for enhanceosome assembly (Yie, et al., 1997). HMG I(Y) binding has been shown to alter the DNA structure of the IFN-β promoter by unbending an intrinsic bend in the promoter (Falvo et al., 1995). Direct contact between HMG I(Y) and the transcription factors is also required for the completion of enhanceosome assembly (Yie et al., 1997, 1999).

In the case of the IL-2 gene, functional studies using antisense expression for HMG I(Y) RNA showed that HMG I(Y) was a positive activator of the IL-2 promoter (Himes et al., 1996, 2000). *In vitro* studies showed that HMG I(Y) could affect the binding of many of the transcription factors involved in IL-2 transcription including NFAT, NF-kB and AP-1 (Himes et al., 1996, 2000; Shang et al., 1999). The non-DNA binding mutant of HMG I(Y) acted as a dominant-negative protein inhibiting the activity of the IL-2 promoter (Himes et al., 2000). This implies that the DNA binding capacity of HMG I(Y) is important for transcriptional activity. Experiments on the IFN-β promoter have also shown that both DNA binding and HMGI(Y):transcription factor interactions are important for the formation of a functional IFN-β enhanceosome (Yie et al., 1999).

HMG I(Y) has also been shown to play an important role in IL-4 gene expression. There are several HMG I(Y) binding sites in the IL-4 promoter (Chuvpilo et al., 1993). These sites are associated with functional binding sites for NFAT or NFAT/AP-1 complexes and Oct proteins (Chuvpilo et al., 1993). It has been shown that HMG I(Y) inhibits the binding of NFAT factors to one specific region of the IL-4 promoter (Klein-Hessling et al., 1996). When the HMG I(Y) binding site was mutated, leaving the NFATp binding site intact, an increase in IL-4 promoter activity was observed. The relative levels of NFATp and HMG I(Y) may be important in determining the functional outcome of the interaction between NFATp and HMG I(Y).

HMGI(Y) has been shown to inhibit transcription from the germline immunoglobulin epsilon (Igε) gene (Kim et al., 1995). It is interesting that this gene is induced by IL-4 and thus may share some similarity with the IL-4 promoter itself. It was found that IL-4 treatment of cells could lead to serine phosphorylation of HMG I(Y) (Wang et al., 1995), which could, in turn, lead to an increase in transcription from those genes that are repressed by HMGI(Y) such as IL-4 or Igε. On the other hand, promoters of genes such as IL-2 or GM-CSF, that are activated HMG I(Y), may have decreased activity after HMG I(Y) phosphorylation (Shannon et al., 2001).

1.5.4. IL-5 gene regulation

1.5.4.1. Activation of the IL-5 gene

The expression of IL-5 is closely linked with the production of eosinophils. These blood and tissue leukocytes are produced in a limited range of immune responses, indicating a unique control of the production of IL-5 in the T-cell response to antigen. Clearly this control is regulated at the level of gene expression. Considering the tight

inducible control of IL-5 expression, as well as the similarity in the development of eosinophilia in different species, it might be expected that the specific regulatory elements involved in its expression would be highly conserved. It is interesting in this context that the flanking regions of the human and mouse genes have very limited homology. The region –80 to –20 is highly conserved, but upstream and 3' regions give little indication of common elements that might be involved in the specific regulation of the gene. The conserved proximal region appears critical for inducible expression, but as discussed below, other elements outside this region are probably also required for overall regulation.

Activation of T-cells requires both interaction of the T-cell receptor (TCR) complex with antigens in association with the major histocompatibility complex and co-stimulation generated by the CD28 signalling pathway. Activation of the TCR results in an increase in intracellular calcium concentration and activation of protein kinase C (PKC) (Perlmutter et al., 1993). *In vitro* activation of T cells can be achieved by stimulating the TCR with anti-CD3 mAb. The TCR-CD3 pathway is sensitive to CsA and FK506. Stimulation of the TCR alone, however, is insufficient to fully activate most T cells and a second signal provided by antigen presenting cells (APC) is also required (Rincon and Flavell, 1994). This costimulatory signal can be generated through the CD28 signaling pathway (Jenkins et al., 1993). Interestingly CD28 signal transduction is resistant to CsA but is sensitive to rapamycin (Lai and Tan, 1994). Commonly used agents which mimic T cell activation are PMA and ionomycin (a Ca^{2+} ionophore used to raise intracellular Ca^{2+} levels).

A widely used cell line for studies on IL-5 regulation is the T cell leukemia line EL-4. IL-5 expression has been shown to be strongly stimulated by the combination of PMA

and cAMP in these cells (Lee et al., 1993; Lee et al., 1994a). Thus it appears that optimal induction of the mouse IL-5 gene in EL-4 cells requires two signals, one generated by PMA which activates PKC (Nishizuka, 1986) and the other by cAMP which exerts its action through the PKA pathway (Lee et al., 1993). It is important to note that while cAMP markedly enhances the expression of IL-5, it has an inhibitory effect on expression of IL-2, IL-3, and GM-CSF (Lee et al., 1993, Chen and Rothenberg., 1994; Derigs et al., 1990) and also no effect on IL-4 (Novak and Rothenberg, 1990).

Studies of IL-5 gene activation compared with a number of other cytokine genes have also been carried out with the mouse Th2 clone D10.G4.1 (Naora et al., 1994b, Naora et al.,1994a). The regulation of the IL-5 gene was shown to be primarily at the level of transcription and to involve *de novo* protein synthesis. Expression of the IL-5, IL-6 and IL-10 genes induced by ConA was resistant to CsA whereas IL-3 and IL-4 expression was completely inhibited. In keeping with this, the IL-5, IL-6 and IL-10 genes were not induced by Ca^{2+} ionophore in contrast to the IL-3 and IL-4 genes. The IL-5, IL-6 and IL-10 genes were induced by IL-1α, PMA and forskolin (an activator of adenylate cyclase) and Abs to CD2 and CD45. The effect of inhibitors suggested that IL-5 gene expression was dependent on PKC and PKA activation (Naora et al., 1994a). Similar comparative studies were carried out using EL-4 (Naora et al., 1994b).

Cell systems suitable for studying human IL-5 regulation have been limited and the mouse EL-4 line has been used in the study of human IL-5 regulation (Gruart-Gouilleux et al., 1995). The human Jurkat T cell line has also been used in some cases (Blumenthal et al., 1999) but has the disadvantage that it does not express IL-5 when stimulated and therefore must be lacking part of the regulatory apparatus for normal IL-5 induction. The human leukemia T cell line HSB-2 is a potential model for regulation studies. Activation

of the human IL-5 gene in these cells can be achieved with PMA/ionomycin stimulation and expression is repressed by CsA and FK506 (Rolfe et al., 1997). IL-5 expression in this cell line is therefore similar to that in normal T cells (Rolfe et al., 1992). Recently, the PER-117 human T cell leukemia line has been shown to be a useful model for IL-5 regulation studies (Mordvinov et al., 1999) but this cell line has not been generally available.

1.5.4.2. Transcription regulation of the mouse IL-5 gene

Studies on the regulation of the mouse IL-5 gene have used a variety of promoter constructs of varying lengths carrying up to 3859 base pairs of upstream sequence. To simplify discussion, it is useful to define the proximal promoter region as the region -1 to -88. This region seems to have particular significance in IL-5 gene induction and is the main focus of the work described in this thesis.

Previous studies in this laboratory using stable transfection of D10W cells (a faster growing derivative of the Th2 clone D10.G4.1) showed that a reporter construct carrying 1170 bp upstream sequence could be induced over 100-fold when the cells were stimulated by PMA/cAMP and was repressed by dexamethasone (Tan, 1998; Young et al., 1999). Deletion studies showed that the proximal promoter region gave similar high levels of inducibility which was Th2 specific (Tan, 1998; Young et al., 1999). No major positive control elements upstream of the proximal promoter region were detected by deletion studies. The expression directed by the proximal promoter region was found not to be repressible by dexamethasone indicating upstream elements involved in this property. In contrast to the results of Tan (1998), a variety of elements in the upstream region or at the 3' end of the gene have been reported by other laboratories as affecting IL-5 expression but there is considerable disagreement over the results. Most are positive

elements. These include: mPRE1-IL5 (-90/-79, Schwenger et al., 1998); IL-5RE-B (-96/-76, Stranick et al., 1998); IL-5P (-117/-92, Lee et al., 1995) which binds NFATp, c-Fos and c-Jun; IL-5PRE (-224/-81, Stranick et al., 1995); mPRE2- IL5 (-470/-459, Schwenger et al., 1998); mDRE (3' end of gene, Salerno et al., 2000) which binds Oct1 and Oct2. Some negative elements have also been reported including: NRE-II (-300/-261, Stranick et al., 1995); NRE-I (-431/-392, Stranick et al., 1995); CTF/NF1 (-940/-928, Bourke et al., 1995). As yet there is little consensus on the importance of the upstream elements in regulating IL-5 expression.

There is more consensus and, in general, more data available for the role of the regulatory elements in the proximal promoter region. The proximal promoter region of the IL-5 gene carries a region which is highly conserved in the human and mouse IL-5 and GM-CSF genes, and to a lesser extent in the IL-3, IL-4 and G-CSF genes (Campbell et al., 1988; Miyatake et al., 1991). This region is referred to as the CLE0 (conserved lymphokine element 0) element (Miyatake et al., 1991) which is located at -53 to -39 in the mouse IL-5 promoter. CLE0 has been shown to be a positive regulatory element in both IL-5 (Naora et al., 1994a; Lee et al., 1995; Bourke et al., 1995) and GM-CSF (Masuda et al., 1993; Fraser et al., 1994). The CLE0 element is essential for the induction of the GM-CSF promoter by PMA and Ca^{2+} ionophore through a calcineurin-mediated pathway (Miyatake et al., 1991; Nimer et al.,1990; Tsuboi et al., 1994). Deletion analysis of the mouse IL-5 promoter in EL-4 cells showed the CLE0 element was critical for IL-5 expression. Mutations in this element almost completely abolished expression in response to PMA and PMA/cAMP (Lee et al., 1995). Similarly, the importance of this element was demonstrated by the establishment of stable transfectants in EL-4 and D10 cells. Mutation of CLE0 markedly reduced expression of the gene in D10 cells induced by PMA or ConA (Naora et al., 1994b) and in EL-4 cells when induced by PMA (Bourke et

al., 1995). Support for the role of CLE0 in D10 cells has also come from the work of Stranick et al., (1998). In these studies, proteins binding to the CLE0 element were generally found to be inducible with little binding activity in nuclear extracts from unstimulated cells. Proteins of similar mobility were noted in an EL-4 subline which did not produce IL-5, as well as in the human Jurkat cell line which does not produce IL-5. Thus induction of the protein complexes binding to the CLE0 element appears to be essential for expression but is not specifically related to the expression of the IL-5 gene (Karlen et al., 1996). However, Stranick et al., (1995) reported that the protein complexes binding to the CLE0 element were expressed constitutively and that the CLE0 element contributed to but was not essential for IL-5 reporter expression in antigen-stimulated D10.G4.1 cells.

The CLE0 element has been recognized to consist of putative AP-1 and Ets/NFAT binding sites which are adjacent. Binding to the AP-1 site has been relatively well studied but binding to the Ets/NFAT site has not been detected. The major inducible proteins binding to the AP-1 region of the mouse IL-5 CLE0 element have been reported to be JunB and JunD in D10 cells (Zhang et al., 1997), c-Fos , JunB and JunD in EL-4 cells (Siegel et al., 1995), and c-Fos, JunB and NFAT in EL-4 cells (Karlen et al., 1996). More recently it has been demonstrated that the immediate 5' end of the CLE0 element carries an Oct site which overlaps with the AP-1 site (Salerno et al., 2001).

There are several reports suggesting the importance of a GATA site (-71 to -66) located upstream of the CLE0 element in regulating IL-5 expression. These include studies in EL-4 cells (Siegel et al., 1995; Lee et al., 1995) and in D10.G4.1 (Zhang et al., 1997, 1998; Lee et al., 1998; Stranick et al., 1998). Siegel et al., (1995) and Zhang et al., (1997) showed that GATA-3 rather than GATA-4 bound to GATA element. GATA-3 has

been shown to be specifically expressed in Th2 cells and to be important in the expression of Th2-specific cytokines (Zheng and Flavell, 1997; Zhang et al., 1998). The proximity of the GATA element to CLE0 suggests the possibility that these elements and the factors interacting with them work in concert in regulating IL-5 gene induction.

1.5.4.3. Transcriptional regulation of the human IL-5 gene

Van Straaten et al., (1994) showed that ConA or PMA but not Ca^{2+} ionophore could stimulate IL-5 expression in human peripheral T cells. The expression of IL-5 could also be blocked by inhibition of protein synthesis. These results show a strong similarity with studies of mouse IL-5 gene expression (Naora and Young, 1995). The CLE0 element and GATA site in the proximal promoter region of the IL-5 gene is highly conserved between mouse and human. However, studies with the human IL-5 promoter in mouse EL-4 cells showed that deletion to –67, which would delete the GATA site, had no effect on IL-5 expression (Gruart-Gouilleux et al., 1995). Also, studies of human IL-5 expression in mouse D10 cells demonstrated that deletion to –80 increased the constitutive expression and removed inducibility (Stranick et al., 1997), suggesting that the CLE0 element, which is left intact, is not responsible for inducible expression. Such results are in contrast to the demonstrated roles of CLE0 and the proximal GATA site in mouse IL-5 expression. On the other hand, Yamagata et al., (1995, 1997) showed that the GATA site has a significant effect on human IL-5 expression when tested in the ATL-16T cell line which expresses IL-5 constitutively and also demonstrated a role for the CLE0 element. The transactivator protein Tax appeared to stimulate expression via the CLE0 element (Yamagata et al., 1997; Blumenthal et al., 1999). One puzzling feature of these studies, which remains controversial, is the demonstration of a selective role for GATA-4 in binding to the GATA site and functioning in IL-5 gene expression. Studies with Jurkat T

cells suggested that Ets1 or Ets2 could function at the Ets/NFAT site of the CLE0 element (Blumenthal et al., 1999).

The above studies either used the mouse cell line EL-4 or human cell lines which do not express the IL-5 gene in a fashion analogous to normal T cells. More recently, the human T cell leukemia line PER-117 has been shown to be a suitable model for IL-5 expression studies (Mordvinov et al., 1999) and data obtained supporting the involvement of CLE0 in human IL-5 expression (Thomas et al., 1999). Binding of Oct factors to the region -67 to -30 of the human IL-5 promoter, which includes the CLE0 element, was demonstrated (Gruart-Gouilleux et al., 1995), suggesting a role for this group of transcription factors in the activation of IL-5. Oct1 and Oct2 have been reported to be involved in human IL-5 gene activation in the PER-117 cell line. One of the binding sites is the CLE0 element (Thomas et al., 1999).

There have also been several regulatory elements reported which are outside of the proximal promoter region of the human IL-5 gene. These include the positive elements: REII (-123/-100, Stranick et al., 1997) which binds NFAT and AP-1, REIII (-17-/-130, Stranick et al., 1997) and one at -312/-227 (Gruart-Gouilleux et al., 1995) which binds Oct, and the negative elements: BR3 (-90/-79, Mordvinov et al., 1999) which binds Oct1 and YY1, a GATA site (-400, Schwenger et al., 2001) which binds GATA-3 acting as a repressor and hPRE2-IL-5 (-459/-447, Schwenger et al., 1999) which binds NFAT and YY1.

1.6. Aims of the research

It is clear from the above summary that there is still disagreement about the molecular mechanisms involved in regulation of the IL-5 gene. Some of these

disagreements may come from studying IL-5 regulation in cell lines which do not express the endogenous IL-5 gene such as Jurkat cells, or which express IL-5 in an abnormal fashion such as the human T-cell leukemia cell line ATL-16T. The study of human IL-5 constructs in mouse cell lines is also not desirable. The mouse thymoma EL-4 cell line has been widely used to study IL-5 regulation but it is not a T-cell clone and it is therefore desirable to confirm results in Th2 cells such as D10.G4.1. IL-5 has been shown to play an important role in the pathogenesis of allergic diseases such as asthma. Therefore there is a considerable interest in identifying the molecular mechanisms controlling the IL-5 expression. Studies in mouse T cell lines provide a valuable starting point for identification of the basic control mechanisms relevant to IL-5 gene expression which should then be followed up in human T cells if the findings are to be applicable to human allergic conditions.

There have been relatively few functional studies on the identification of transcription factors which are specifically involved in both human and mouse IL-5 gene activation even though several potential regulatory elements have been shown to be important in IL-5 expression. In addition, there has been little clarification of the factors which function at the Ets/NFAT site in the CLE0 element. Also little is known about the MAP kinase pathways activating the IL-5 transcription.

In this study, the mouse D10 Th2 clone cell and the human HSB-2 T cell leukemia line were used to study the transcriptional activation mechanisms of the mouse and human IL-5 genes, respectively. Both cell lines exhibit inducible expression of the IL-5 gene and appear to be good models for studying IL-5 gene regulation.

Specific aims of the research:

1) To establish transient transfection systems in both mouse D10W cells and human HSB-2 cells using the sensitive luciferase reporter gene.

2). To determine the optimum conditions for inducible expression of reporter genes in D10W and HSB-2 cells

3) To investigate the transcription factors functioning at the transcriptional elements of the IL-5 promoter proximal region and to study possible synergistic effects between them using transactivation assays. In addition, to clarify the activity of GATA-3 compared with GATA-4 and whether Ets or NFAT family members function at the CLE0 element.

4) To study the role of MAP kinase pathways in induction of IL-5 gene expression.

Chapter 2 Materials and Methods

2.1. Preparation of bacterial cells

2.1.1. *E. coli* DH5αF'I^{Q}

The main bacterial cell used in this thesis was *E. coli* DH5αF'I^{Q}. This strain from Life Technologies Inc., USA, is a derivative of Hanahan's strain DH5 (Hanahan, 1985). The *lac*ZΔM15 locus is a partial deletion of the β-galactosidase gene that allows α-complementation between the portion of the β-galactosidase gene carried by the pUC or M13-based plasmid and the portion on the chromosome of the *E. coli* host cell, allowing the blue/white screening of clones. The *rec*A1 and *end*A1 alleles greatly enhance the ability to retain recombinant plasmids. The F' episome contains a kanamycin-resistance marker (zzf::Tn5), allowing DH5αF'I^{Q} to be maintained on rich medium (LB or YT) containing kanamycin without loss of the F' episome. The strain also expresses large amounts of the *lac*I repressor due to the *lac*I^{q} locus, thus allowing IPTG induction of cloned sequences under the control of the *lac* promoter.

2.1.2. Electro-competent bacterial cells

A single colony of DH5α bacterial cells was inoculated into 50 ml of LB medium and the culture shaken at 250 rpm at 37°C overnight. 10 ml of overnight culture was used to inoculate 1000 ml of LB medium and the culture grown with shaking at 37°C until the OD_{600} reached between 0.5 and 1.0. The cells were then chilled on ice for 30 minutes and centrifuged at 4°C for 15 minutes at 5000 rpm. The

supernatant was removed and the cells were washed once with 1000 ml of cold MQ water, once with 500 ml of cold MQ water and once with 20 ml of cold water before final resuspension in 3 ml of cold 10% glycerol. Forty µl aliquots of the suspension were dispensed into sterile Eppendorf tubes on ice and stored at -70°C.

2.1.3. Transformation of electro-competent bacterial cells

Ten ng of DNA was mixed with 40 µl of electro-competent cells in an ice-cold micro-cuvette (2 mm path length), The cells were transfected by electroporation using the Bio-Rad Gene Pulser II set at 2.5 KV, a capacitance of 25 µF and with 200 Ohms resistance. The transformed cells were immediately incubated in 1 ml of LB at 37°C for an hour to allow recovery before selection on LB agar plates containing the appropriate antibiotic (usually ampicillin at 100 µg/ml).

2.2. Recombinant DNA techniques

2.2.1. Mini preparation of plasmid DNA

DNA was prepared using the WIZARD DNA Minprep Kit (Promega). according to the manufacturer's instructions. Briefly, cells from an overnight bacterial culture in medium supplemented with antibiotic (eg 100 µg/ml ampicillin) were harvested by centrifugation. and lysed by alkaline lysis. The lysate was neutralised and the cellular debris removed by centrifugation. The supernatant was passed through a silica-based matrix that allowed binding of the DNA. The DNA was subsequently eluted using $T_{10}E_1$ buffer (10 mM Tris-HCl, 1 mM EDTA, pH8.0).

2.2.2. Large scale preparation of plasmid DNA

Large amounts of DNA were prepared using the QIAGEN Maxiprep Kit according to the manufacturer's instructions. For low yielding plasmids, an amplication procedure was carried out prior to using the kit. A single colony of bacterial cells was used to inoculate 20 ml of LB medium supplemented with 100 μg/ml ampicillin and grown overnight at 37°C with shaking at 250 rpm. 10 ml of overnight culture was used to inoculated 300 ml of fresh LB medium supplemented with 100 μg/ml ampicillin and the culture grown with shaking at 37°C until the OD_{600} was between 0.5 and 1.0. Chloramphenicol (170 μg/ml), a protein synthesis inhibitor, was then added and the culture grown under the same conditions overnight. The cells were harvested by centrifugation at 4000 rpm and lysed by alkaline lysis. After removal of the cell debris by centrifugation, the crude DNA was applied to an anion-exchange resin under conditions of low salt. RNA, proteins and low molecular weight components were removed by a medium salt wash. The plasmid DNA was eluted by a high salt buffer, concentrated and desalted by isopropanol precipitation.

2.2.3. Quantitation of nucleic acids

The amount of DNA or RNA in solution was quantitated by measuring its optical density (OD) at 260 nm and the concentration calculated using the relationship that at 260 nm, an OD of 1 is equivalent to 50 μg/ml of double stranded DNA and 40μg/ml of single stranded RNA. The ratio between the OD reading at 260 nm and 280 nm (OD_{260}/OD_{280}) was used to give an estimate of the purity of the nucleic acid preparation. Pure preparations of DNA and RNA have OD_{260}/OD_{280} ratios

of 1.8 and 2.0 respectively, with lower readings being obtained if there is protein or phenol contamination (Sambrook et al., 1989).

2.2.4. Restriction enzyme digestion of DNA

Restriction endonucleases were supplied by New England Biolabs or Boehringer Mannheim. DNA digestion was performed according to the manufacturer's protocols and in the buffer specified for each enzyme. The DNA concentration in the final reaction volume was kept at between 50 to 100 ng/μl. Bovine serum albumin (BSA) was routinely included in the reaction at 0.1 mg/ml.

2.2.5. Electrophoresis of DNA

Agarose gel electrophoresis was used to separate and purify DNA fragments generated by restriction enzyme digestion of plasmid DNA. Electrophoresis was carried out using a voltage gradient of between 1-5 V/cm and using 1 x TAE buffer containing 40 mM Tris-acetate, 1mM Tris-HCl pH 8.0. Prior to loading, the DNA samples were mixed with 0.1 volume of 10 X loading buffer containing 0.25% bromophenol blue, 0.25% xylene cyanol, and 40% sucrose.

2.2.6. Purification of DNA fragments

After agarose gel electrophoresis, the DNA fragment of interest was purified using the Bresaclean Kit (Bresatec Inc., Australia) according to the manufacturer's instructions. Briefly, the electrophoresed DNA fragment was extracted from an agarose gel using a chaotropic salt (NaI) and absorbed to a silica matrix. The matrix was washed to remove impurities. The silica-DNA pellet was resuspended in $T_{10}E_1$

and incubated at 50°C for two minutes. The eluted DNA was recovered by centrifugation.

2.2.7. Dephosphorylation of DNA termini

Following restriction enzyme digestion, the DNA was purifed using Bresaclean Kit (Bresatec, Australia). The pellet was then dissolved in a buffer containing 50 mM Tris-HCl pH8.5, 0.1 mM EDTA and 1 U of calf intestinal alkaline phosphatase (CIP) per 2 pmol of DNA ends was added then the mixture incubated at 37°C for 15 minutes. After that, an equal amount of CIP was added and the incubation continued at 55°C for 45 minutes. The reaction was terminated by the addition of SDS, EDTA pH8.0 and Proteinase K to final concentrations of 0.5%, 5 mM and 100μg/ml respectively and the mixture incubated at 55°C for a further 30 minutes. The DNA was then purified using the Bresaclean Kit. The DNA was then ready for ligation.

2.2.8. DNA ligation

Generally, a 5-fold molar excess of insert DNA to vector DNA was used in cohesive end ligation reactions. For blunt-end ligation a 10- to 20-fold molar excess of insert DNA was used. The ligation reaction was carried out in a 10 μl volume containing 30 mM Tris-HCl pH.8, 10 mM $MgCl_2$, 10 mM DTT, 1mM ATP and 1-3 Weiss units of T4 DNA ligase (Promega). The reactions were incubated at 16°C for 16 hours before termination of the reaction by heating at 65°C for 15 minutes.

2.2.9. PCR analysis of recombinant plasmid clones

Colonies were picked and transferred to 50 µl of MQ water with a sterile toothpick and the suspensions incubated at 65°C for 10 minutes to lyse the bacterial cells. Each colony was also used to inoculate a numbered grid plate. Five µl of lysate was amplified in a 20 µl reaction containing 5 pmol of each of two flanking primers (sense primer on the inserted DNA; anti-sense primer on the vector sequence), 0.2 mM of dNTPs, 1.5 mM $MgCl_2$, 10 mM Tris-HCL, 50 mM KCl, 0.1 units of Taq DNA polymerase (Promega). The PCR reaction was carried out in a Thermal cycler (Corbett, FTS-1) using the following conditions: denaturation at 94°C for 10 seconds, annealing at 65°C for 10 seconds, and extension at 72 °C for 1 minute. This cycling was repeated 35 times, and at the end of the procedure, the PCR products were analysed by agarose gel eletrophoresis.

(I) Primer pairs used for screening mouse IL-5 promoter mutants

Sense primer 5'-ATGGGCGTCTCTAGAGAGATCC-3'

Anti-sense primer 5'-TTATGCAGTTGCTCTCCAGCG-3'

(II) Primer used for screening human IL-5 promoter mutants

Sense primer 5'-GCAAATGTGGGGCAATGATG-3'. This primer binds to the human IL-5 promoter region (-160 to -140) and was used to directly sequence the colonies obtained from the mutagenesis.

2.2.10. Mutagenesis of DNA

The QuickChangeTM Site-directed Mutagenesis system (Stratagene) was used. This system uses *PfuTurbo* DNA polymerase and a thermal temperature cycler. *PfuTurbo* DNA polymerase replicates both plasmid strands with high fidelity and without displacing the mutant oligonucleotide primers. Briefly, the basic procedure uses a supercoiled double-stranded DNA vector with an insert of interest and two synthetic oligonucleotide primers containing the desired mutation. The oligonucleotide primers, each complementary to opposite strands of the vector, are extended during temperature cycling using *PfuTurbo* DNA polymerase. Incorporation of the oligonucleotide primers generates a mutated plasmid containing staggered nicks. Following temperature cycling, the product is treated with *Dpn* I. The *Dpn* I endonuclease (target sequence: 5'-Gm6ATC-3') is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template and to select for the mutation-containing synthesized DNA. DNA isolated from almost all *Escherichia coli* strains is *dam* methylated and therefore susceptible to *Dpn* I digestion. The nicked vector DNA incorporating the desired mutations is then transformed into DH5α competent cells.

Primers for mutagenesis

Primers used for site-directed mutagenesis of the AP-1, Ets and GATA binding sites of the human IL-5 promoter are listed below:

Ets site mutant:

5'-GATTGTTAGAAATTATTCATggaaTCAAAGACAGACAATAAATTG-3'

AP-1 site mutant:

5'-CTCTATCTGATTGTTAGAAATccTatATTTCCTCAAAGACAGAC-3'

GATA site mutant:

5'-CCATTATTAGGCATTCTCTAagTGATTGTTAGAAATTATTCATTTC3'

Primers used for site-directed mutagenesis of the AP-1, Ets/NFAT and GATA sites of mouse IL-5 promoter are listed below:

Ets site mutant:

5'-GATTGTTAGCAATTATTCATggaaTCAGAGAGAGAATAAATTGCT-3'.

AP-1 site mutant:

5'-CTCTATCTGATTGTTAGCAATccTatATTTCCTCAGAGAGAGAA-3'

GATA site mutant:

5'-CCTTTATTAGGTGTCCTCTAagTGATTGTTAGAAATTATTCATTTC-3'

Mutagenesis procedure

Mutagenesis was carried out using the QuickChangeTM Site-directed Mutagenesis system (Stratagene) according to the manufacturer's instructions. Briefly, the reaction mixture consisted of 125 ng of each of two complementary primers containing the desired mutation as listed above, 50 ng of double stranded plasmid template, 50 μM dNTPs, 1.5 mM MgCl2, 10 mM Tris-HCl pH9.0, 50 mM KCl, and 2.5 units *PfuTurbo* DNA polymerase in a final volume of 50 μl. The

following program was used: 1 cycle of 30 seconds at 95°C; 18 cycles of 30 seconds at 95°C (denaturation), 1 minute at 55°C (annealing), 14 minutes at 68°C (extension); and 1 cycle of 7 minutes. After mutagenesis reaction,10 units of *Dpn* I was added, the reaction mixture incubated at 37 °C for 1 hour and then 1 µl of reaction mixture was used for transformation. After transformation, individual colonies or pools of colonies were picked and screened by PCR. Mutants were sequenced to verify that the desired mutation had been introduced into the target gene.

2.3. Electrophoretic mobility shift assays (EMSA)

2.3.1. Preparation of oligonucleotide probes

Oligonucleotide probes corresponding to various transcription factor binding sites on the proximal promoter region of IL-5 were synthesised by the Biomolecular Resource Facility, JCSMR. The primers corresponding to those regions are listed below. Complementary primers were annealed by combining equimolar amounts of the primer pairs and incubating at 65°C for 10 min. The primers were further heated at 100°C for 5 minutes and the mixture allowed to slowly cool to room temperature in the water bath and stored at -20°C.

2.3.2. Labelling of oligonucleotide probes

One pmol of primer was labelled with 20 µCi of γ-32P ATP (3000 Ci/mmol, GeneWorks, Australia) in a 10 µl reaction with 0.1 units of polynucleotide kinase (BioLabs) in provided buffer. The reaction was incubated at 37°C for 30 minutes. The labelled primers were separated from unincorporated nucleotides on an 8%

acrylamide (acrylamide-bisacrylamide, 29:1) 1 x Tris-borate-EDTA (TBE) gel. The gel was autoradiographed (Kodak) for 1 minute and the fragments were excised from the gel and eluted in an elution buffer containing 0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, pH 8.0 and 0.1% SDS by incubation overnight at 37°C with shaking. The radiolabelled primers were ethanol-precipitated, the pellets washed with 70% ethanol and dried under vacuum followed by resuspension in 12 µl $T_{10}E_1$ pH 8.0. The radioactivity of 1µl of the DNA was measured using a liquid Scintillation Analyser (Packard, 1900CA) and the primers diluted to 300000 cpm/µl.

The following oligonucleotide probes were used for EMSA:

Proximal promoter region of the mouse IL-5 gene:

P1: wt mIL-5 promoter 5'-TTAGCAATTATTCATTTCCTCAGAGAGAG-3'

P2: IL-5 AP-1 mutant 5'- TTAGCAATccTatATTTCCTCAGAGAGAG-3'

P3: IL-5 Ets mutant 5'-TTAGCAATTATTCATggaaTCAGAGAGAG-3'

P4: AP-1 consensus 5'-CGCTTGATGACTCAGCCGGAA-3'

P5: Ets consensus 5'-GATCGAGAGCGGAAGCGCGCGATC-3'

P6: Ets consensus mutant 5'-GATCGAGAGCaGAAGCGCGCGATC-3'

P7: non-specific sequence 5'-CTCATACACACAGTACGCTGCAACT-3'

2.3.3. Binding assay

Binding reactions were performed in a final volume of 25 µl containing 1µg of poly (dI.dC), 25 mM Hepes pH 7.9, 25 mM NaCl, 10% glycerol, 1mM EDTA, 1mM DTT, 0.025% NP-40). The recombinant proteins and 15000 cpm (1.25 ng) of labelled DNA probes were added to the reaction mixtures on ice and then they were incubated for 10-15 minutes at room temperature. After addition of 3 µl of loading dye containing 0.25% bromophenol blue, 0.25% xylene cyanol, 40% sucrose, the samples were loaded directly onto a pre-electrophoresed 4.5% non-denaturing polyacrylamide 0.5 x TBE gel and electrophoresed at 15V/cm at 4°C. The gels were dried and exposed to X-ray film (Kodak) at room temperature overnight.

In competition experiments, 100-fold molar excess unlabelled oligonucleotides which correspond to the same binding site or other unrelated binding sequences were added to the reactions.

2.4. Preparation of reporter and expression constructs

2.4.1. pXPG luciferase reporter construct

pXPG plasmid was generated by incorporating a novel high-copy origin of replication and a modified luciferase gene into a pXP1-derived vector that efficiently blocks read-through transcription in eukaryotic cells (Bert et al., 2000). pXPG encodes the luciferase gene derived from pGL3 that lacks a peroxisomal targeting sequence, thereby allowing accumulation of luciferase protein in the cytoplasm rather

than in subcellular organelles of transfected eukaryotic cells. pXPG has distinct advantages over pGL3, because it contains SV40 polyadenylation signals that appear to be more efficient at blocking read-through transcription than the synethetic polyadenylation signal present in pGL3. pXPG contains a novel mutation near the origin of replication that increases plasmid copy number in *E.coli.* This mutation alters the -10 sequence in the RNA II promoter of the ColE1 origin of replication from TAATCT to TAATAT. This sequence is a closer match to the consensus -10 element, suggesting that the mutation increases copy number by increasing the rate of transcription of the RNA II replication promoter. Unlike pUC, which reverts to low copy number at 30°C, the pXPG mutation supports a higher copy number at both 37°C and 30°C (Bert et al., 2000).

2.4.2. Mouse IL-5 luciferase reporter construct

The -1170mIL-5Luc reporter construct used in this work contains approximately 1170 bp of sequence preceding the transcription initiation site of the mouse IL-5 promoter. A *Sac*I fragment from -1170SynCATmIL-5 construct which encompasses the -1174 to +26 of the 5'-unstranslated region of the mouse IL-5 gene was cloned into the *Sac*I site of the pXPG luciferase reporter construct. The mutations of AP-1, Ets/NFAT and GATA sites of IL-5 promoter were made in this construct.

The -88mIL-5Luc construct which carries the proximal 88 bp of the mIL-5 promoter was generated by PCR. Addition of a *Kpn* I site on the sense primer and a *Bam* HI site on the anti-sense primer allowed the fragment to be subcloned into the pXPG luciferase reporter plasmid. The construct was verified by sequencing.

2.4.3. Human IL-5 luciferase reporter construct

The -1.2khIL-5Luc construct which carries 1200 bp of the upstream region of the human IL-5 promoter was provided by Boehrimger-Ingelheim. This vector is based on pXP1 which was described previously (Nordeen, 1988).

2.4.4. Mammalian expression constructs

2.4.4.1. pEFIRES-p expression plasmid

pEFIRES-p consists of the human elongation factor 1α (EF-1α) promoter driving transcription of a chimaeric intron, multiple cloning site, internal ribosome entry sites (IRES) and the puromycin N-acetyltransferase (*pac*) resistance marker gene as a single mRNA species. This plasmid combines the advantages of a bicistronic message with EF-1α promoter to drive high level protein expression. Details of this plasmid have been described elsewhere (Hobbs et al., 1998).

2.4.4.2. Expression plasmid for mouse GATA-3

2056 bp cDNA encoding GATA-3 was released by *EcoR* I from pGM mc5bB (Ko and Engel, 1993). This fragment was cloned into the *EcoR* I site of pEFIRES-p expression plasmid.

2.4.4.3. Expression plasmid for mouse GATA-4

1799 bp cDNA encoding GATA-4 was released by *EcoR* I from pMT2mGATA-4 plasmid (Bielinska and Wilson, 1995). This fragment was cloned into *EcoR* I site of pEFIRES-p expression plasmid.

2.4.4.4. pEFBOS expression plasmid

pEFBOS carries the SV40 replication origin, human EF-1α promoter, the stuffer fragment from CDM8 vector and poly (A) adenylation signal from human G-CSF. The size of pEFBOS is 5.8 kb and the cDNA to be expressed can be inserted at *Bst* XI site using *Bst* XI adapters, or at the *Xba* I site using *Xba* I linkers. Details of this plasmid have been described elsewhere (Mizushima and Nagata, 1990).

The c-Jun, c-Fos, Ets1 and Elf1 expression constructs were made using pEFBOS expression vector under the control of the EF-1α promoter and were provided by I Kola (Monash University, Australia). The construct for dominant negative AP-1 was provided by C. Vinson (National Institutes of Health, Bethesda, USA).

NFATp and NFATc expression plasmids were provided by F. Shannon (JCSMR, ANU) These two expression plasmids were made using pREP4 expression vector derived by RSV promoter. Details of these plasmids were described elsewhere (Hoey et al., 1995).

2.4.4.5. Plasmids for MAP kinase pathways studies

The p4 x AP-1/Ets construct carries four copies of a combined AP-1/Ets binding site of polyoma virus enhancer in front of a luciferase gene (Hoffmeyer et al., 1999) and provided by Ulf R. Rapp (Universität Würzburg, Germany).

The expression vector Raf-BXB-CX encodes activated Raf which lacks the N-terminal negative regulatory domain and contains the C-terminal membrane targeting 17 amino acids of Ki-Ras fused to the kinase domain of c-Raf. This construct is driven by the RSV-promoter of pRSPA (Hoffmeyer et al., 1998) and provided by Ulf R. Rapp (Universität Würzburg, Germany).

The expression vector MLK3 is a activator of JNK and provided by Ulf R. Rapp (Universität Würzburg, Germany).

The expression vector MKK6E is a specific activator of p38. This vector uses the RSV-promoter of pRSPA (Hoffmeyer et al., 1998) and provided by Ulf R. Rapp (Universität Würzburg, Germany).

2.5. DNA Sequencing

All plasmid constructs generated in this study were verified by sequence analysis. DNA sequencing was performed using the ABI cycle sequencing system following the manufacturer's instructions using an Applied Biosystems Model 373A Automated DNA Sequencer. The sequencer was operated by the Biomolecular Resource Facility, JCSMR.

2.6. Cell culture

2.6.1. D10.G4.1 and D10W cells

The mouse CD3+, CD4+, CD8- T-cell clone D10.G4.1 is a conalbumin-specific, H-2 Iak restricted Th2 clone derived from AKR/J mice (Kaye et al., 1983) and was obtained from Dr. T. Mosmann (DNAX Research Institute, Palo Alto, CA). D10.G4.1 cell growth was induced by allogeneic stimulation every 10-14 days with a 10-fold excess of irradiated spleen cells from 6-8 week old C57B1/6 female mice as accessory antigen presenting cells. D10.G4.1 produces significant levels of IL-5 mRNA and protein after TCR-mediated stimulation, while IL-5 mRNA or protein can

not be detected in the resting D10.G4.1 cells (Naora et al., 1994a; Stranick et al., 1995).

D10W is a spontaneously derived subline of D10.G4.1 produced in this laboratory which grows more vigorously and still shows inducible expression of the IL-5 gene. IL-5 gene induction is also still repressed by dexamethasone. D10W cells appear to be less dependent, but not completely independent of TCR stimulation for growth. The conditions used for the growth and maintenance of D10W cells were the same as those for D10.G4.1 cells except that stimulation using irradiated spleen cells was not carried out.

2.6.2. Human HSB-2 cells

HSB-2 cells express CD2 surface receptors, which are a phenotypic marker of T lymphocytes but lacks CD3 and CD25 surface receptors. The induction of IL-5 mRNA expression in HSB-2 cells occurs in response to stimulation with PMA and ionomycin (Rolfe et al., 1997; Valentine and Sewell, 1998) and IL-5 expression is repressed by CsA or FK506 as with normal human T cells (Rolfe et al., 1997; Rolfe et al., 1992). HSB-2 was provided by W. Sewell (St. Vincent's Hospital, NSW, Australia).

2.6.3. Maintenance of tissue culture cells

Mouse Th-2 type D10W cells and human HSB-2 cells were maintained in RPMI-1640 medium with the following additives: 10% fetal calf serum, 10 mM NaCl; 1 mM sodium pyruvate; 56.2 mM monothioglycerol; 60 μg/ml benzylpenicillin (Commonwealth Serum Laboratories [CSL], Australia); 100 μg/ml streptomycin

(Sigma, St Louis, MO); 2 mM L-glutamine (CSL); 0.05 mM ß-mercaptoethanol (Sigma). For D10W, 25 units/ml of IL-2 were added. The IL-2 was prepared from Sf9 cells infected with a baculovirus expressing mouse IL-2 and was provided by S. Ford (JCSMR). One unit of IL-2 bioactivity is the amount of IL-2 required to support half-maximal proliferation of HT-2 cells. The medium was replaced with fresh medium containing IL-2 every three days. Both D10W and HSB-2 cells were grown at 37°C and 95% relative humidity.

2.7. Transfection assay

2.7.1. Optimization of transfection efficiency

An expression plasmid containing the gene encoding green fluorescent protein (GFP) was used to optimize transfection efficiency in D10W and HSB-2 cells. 5 x 10^6 D10W or HSB-2 cells were mixed with 5 µg of GFP plasmid in 300 µl of RPMI-1640 supplemented with 20% FCS in electroporation cuvettes for 15 minutes at room temperature and subjected to voltage pulses ranging from 230 V to 310 V for D10W cells (220 V to 320 V for HSB-2 cells) at 975 µF capacitance in a GenePulser apparatus (Bio-Rad). Transfected cells were cultured in 5 ml of complete RPMI-1640 medium for 20 hours. One ml of cells were transferred into FACS analysis tubes and stained with PI (Propidium iodide) at 10 µg/ml for 30 minutes, and then subjected to FACS assay. Both total number of dead cells and viable cells expressing GFP were determined. The conditions giving the best transfection efficiency of D10W or HSB-2 cells were established.

2.7.2. Stimulation of the cells

In these studies following chemicals have been used to treat the cells.

Phorbol myristate acetate (PMA) (Sigma): The stock solution of 10 ng/μl was made up in DMSO and stored in the dark at -20°C. The final concentration used was 25ng/ml.

Dibutyryl cyclic AMP (cAMP) (Sigma): The stock solution of 0.1M was made up in MQ water and stored in the dark at -20°C. The final concentration used was 1 mM.

Ca^{2+} ionophore (A23187) (Sigma): The stock solution of 10mM was made up in DMSO and stored in the dark at -20°C. The final concentration used was 1mM.

PD98059 (Calbiochem): a inhibitor of MAP kinase kinase (MEK). The stock solution of 20 mM was made up in DMSO and stored in the dark at -20°C.

SB203580 (Calbiochem): a specific inhibitor of p38 kinase. The stock solution of 10 mM was made up in DMSO and stored in the dark at -20°C.

2.7.3. Time course of IL-5 luciferase reporter gene expression

Five μg of -1170mIL5Luc or -1.2khIL-5Luc plasmid were transfected into D10W or HSB-2 cells according to the established conditions. Transfected cells were treated with PMA and cAMP or ionophore after periods of growth ranging from 0 hour to 24 hours. The cells were harvested and luciferase assays were carried out as described below. Suitable times at which to measure for IL-5 reporter gene expression in D10W and HSB-2 cells were then determined.

2.7.4. Transfection

D10W or HSB-2 cells were grown in RPMI-1640 completed medium and sub-cultured at 5 x 10^5 cells/ml the day before transfection. The cells were harvested and re-suspended in the growth medium described above. 5 x10^6 cells were incubated with 20 μg of DNA (5μg of IL-5 reporter plasmids, 5 μg of different expression plasmid, and empty plasmid to make up to 20 μg of total DNA) in 300 μl of growth medium supplemented with 20% FCS for 15 minutes at room temperature, and subjected to electroporation at 270 V and 975 μF capacitance for D10W cells or 280 V and 975 μF capacitance for HSB-2 cells (Bio-Rad GenePulser). The cells were transfered into 5 ml of fresh medium and incubated at 37°C for 20 hours.

Transfected D10W cells were treated with or without PMA and cAMP for 9 hours. In MAP kinase pathway studies, transfected cells were preincubated with MAP kinase inhibitor for 30 minutes before stimulation. Transfected HSB-2 cells were treated with PMA, cAMP and ionophore for 9 hours (25 ng/ml PMA, 1 mM cAMP and 1 mM ionophore). In the stimulation procedures, DMSO controls were carried out.

2.7.5. Luciferase assays

After 9 hours stimulation, the cells were harvested by centrifugation at 1500 rpm for 5 minutes, washed twice in PBS and then lysed with three rounds of freeze-thaw lysis in liquid nitrogen in a lysis buffer containing 10 mM K_2HPO_4, 1 mM EDTA and 0.2 mM DTT. The debris was removed by centrifugation at 3000 rpm for 10 minutes. Protein concentrations of lysates were determined using the Bio-Rad

protein assay according to the manufacturer's instructions. In the luciferase assays, 15 μg of protein for D10W cell extracts or 30 μg of protein for HSB-2 cell extracts were dispensed into 96 well plates and mixed with 200 μl of assay buffer containing 100 mM potassium phosphate buffer (combine four parts 100mM K_2HPO4 with one part 100 mM KH_2PO4), 8 mM $MgSO_4$, 2 mM DTT, 0.75 mM ATP and 0.175 mM co-enzyme A. The reactions were initiated by adding 40 μl of 1 mM D-luciferin. The light signal was measured using TopCounter (Packard). The linearity of the assay was confirmed for high activity samples by measuring the activity of two-fold dilutions of the protein samples. The background of assay was very low and taken away from all values. Mean and standard deviation of at least three independent experiments are shown in the figures.

Chapter 3 A Primary Role for AP-1, in Synergy with Ets1 and GATA-3 in the Inducible Expression of the IL-5 Gene in Mouse Th2 Lymphocytes

3.1. Introduction

Previous studies in this laboratory showed that the regulation of the mouse IL-5 gene in the Th2 clone D10.G4.1 was primarily at the level of transcription. Induction of IL-5 gene expression could be achieved by stimulation of the TCR, by ConA or by agents stimulating PKC and PKA (Naora et al., 1994b; Naora and Young, 1995). In each case there was an absolute requirement for protein synthesis (Naora and Young, 1995). Subsequently, stable transfection studies with the IL-5 gene in a normal chromatin environment were carried out using CAT reporter genes. A reporter construct carrying 3859 bp of the upstream sequence of the mouse IL-5 gene was shown to have the same expression characteristics as the endogenous IL-5 gene (Bourke et al., 1995). It was found that a shorter construct (-1062mIL-5CAT) also showed the same expression properties (Naora et al., 1994a; Bourke et al., 1995). An analysis of the proximal promoter region of the mouse IL-5 gene revealed two major regions of transcription factor binding sites called mIL-5A (-60 to -40) and mIL-5B (-76 to -61) (Naora, 1993). Immediately upstream of the TATA box at positions -41 to -55 in the mIL-5A region is the CLE0 site (conserved lymphokine element 0) (Miyatake et al., 1991). The CLE0 element contains an AP-1 like binding site adjacent to a putative Ets/NFAT site. There is a putative GATA site within the mIL-5B region at -67 to -72. Site-directed mutagenesis showed that each of the three transcription factor binding sites was essential for IL-5 expression (Naora et al.,

1994a; Tan, 1998; Young et al., 1999). In addition, it was shown that the proximal promoter region of the mouse IL-5 gene is responsible for its tissue-specific inducible expression in Th2 cells (Bourke et al., 1995; Tan, 1998; Young et al., 1999).

A number of studies on mouse IL-5 gene expression have been carried out in other laboratories using transient transfection (see Chapter 1). Although there is considerable disagreement in the findings, several studies have supported an important role for the CLE0 element (Naora et al., 1994a; Lee et al., 1995; Bourke et al., 1995; Stranick et al., 1998) and for the adjacent GATA element (Lee et al., 1995, 1998; Siegel et al., 1995; Stranick et al., 1998; Zhang et al., 1997, 1998). The CLE0 element carries an atypical AP-1 site adjacent to a site which could potentially involve a member of the Ets or NFAT families. Recently, Oct binding to the CLE0 site has been demonstrated (Salerno et al., 2001). The Oct site is immediately upstream of and slightly overlapping with the AP-1 site. Although GATA-3 is the more likely transcription factor to be active at the GATA site due to its selective expression in Th2 cells, a selective role for GATA-4 has been reported for human IL-5 expression (Yamagata et al., 1995, 1997). One way of further investigating the transcription factors involved in IL-5 expression is transactivation studies using transient transfection of expression constructs.

The purpose of the work described in this Chapter was to establish a transient transfection assay utilizing the luciferase reporter gene system in the Th2 clone D10W to enable analysis of the functional role of the GATA, AP-1 and Ets/NFAT regulatory elements in the proximal promoter region and to further identify the transcription factors that may be involved in the regulation of the IL-5 gene by transactivation studies.

3.1.1. GATA transcription factor

The GATA family of zinc finger transcription factors plays an important role in regulating cell lineage differentiation during vertebrate development. Six GATA family members have been identified in vertebrate species. These six proteins have been classified into two subfamilies based on common amino acid sequence identities and patterns of expression (Weiss and Orkin, 1995). Each GATA family member contains a conserved C-X2-C-X17-C-X2-C (type IV) zinc finger DNA-binding domain that recognizes a consensus sequence motif (WGATAR) located within transcriptional regulatory elements that control sets of lineage-specific genes (Ko and Engel, 1993; Merika and Orkin, 1993).

GATA-3 was initially cloned as a T-cell-specific transcription factor that bound to the Ta3 element of the TCRα gene enhancer (Ho et al., 1991). Deletion of the GATA-3 gene produces lethal abnormalities of the central nervous system and hematopoietic system (Pandolfi et al., 1995), whereas mice lacking GATA-3 in the lymphoid system do not generate T cells because GATA-3-deficient thymocytes arrest at the immature double-negative stage (Ting et al., 1996; Hendriks et al., 1999). A key discovery was the selective expression of GATA-3 in Th2 cells. Ectopic expression of a GATA-3 transgene led to increased levels of Th2 cytokines, whereas a dominant-negative GATA-3 transgene inhibited Th2 differentiation (Zheng and Flavell, 1997; Zhang et al., 1999). Several reports support a role for GATA-3 in the control of IL-5 gene expression through binding to and transactivation of elements in the region -70 to -59 of the IL-5 promoter (Siegel et al., 1995; Zhang et al. 1997, 1998). GATA-3 probably does not directly bind to and transactivate the IL-4 promoter (Zhang et al., 1997, 1998). Rather, several genomic regions within the IL-

4/IL-13 locus have GATA-3-dependent enhancer activity (Ouyang et al., 1998) suggesting GATA-3 may augment expression of IL-4 or IL-13 via interactions at sites distant from the proximal promoter.

In addition to increasing expression of Th2-selective cytokines, GATA-3 inhibits Th1 development independently of IL-4, perhaps in part by inhibition of IL12Rβ2 expression (Ouyang et al., 1998). Although GATA-3 is a Stat6-inducible gene, the developmental program initiated by GATA-3 can operate independently of Stat6 (Ouyang et al., 2000). When GATA-3 was expressed in Stat6-deficient T cells, all components of Th2 development were observed, including induction of Th2 cytokines, inhibition of Th1 cytokines, induction of c-Maf, and the formation of DNase I hypersensitive sites in the IL-4 locus. The endogenous GATA-3 gene was observed to be induced in these cells, despite the addition of IL-12 to the culture, normally a signal that inhibits GATA-3 expression (Ouyang et al., 1998). These findings suggest that GATA-3 plays a key role in promoting Th2 differentiation and that GATA-3 expression is suppressed in developing Th1 cells.

3.1.2. Ets transcription factor

The v-Ets oncogene was originally discovered as part of a fusion protein expressed by a transforming retrovirus, and later shown to be transduced from a cellular gene (Nunn et al., 1983). The v-Ets protein contains an 84 amino acid sequence, the" Ets domain" that has been found in proteins from species ranging from *Drosophila* to humans (Karim et al., 1990). Ets proteins have been implicated in the regulation of gene expression during a variety of biological processes, including

growth control, transformation, T-cell activation and developmental programs in many organisms (Macleod and Plumb, 1991). The expanding family includes Ets1 (Gegonne et al., 1987), Ets2 (Boulukos et al., 1988), Erg (Reddy et al., 1987) Elk-1 and Elk-2 (Rao et al., 1989), Spi 1/PU.1 (Klemsz et al., 1990) Fli-1 (Ben-David et al., 1991), Elf1 (Leiden et al., 1992), the Drosophila E74A and E74B proteins (Burtis et al., 1990) and the α-subunit of the GA-binding protein (GABP) (LaMarco et al., 1991). The Ets family can be classified into subfamilies, based on sequence similarities in the Ets domain, the position of the Ets domain in the protein, and additional sequences found only in sub-families (Wasylyk et al., 1993). The Ets proteins have been found to bind specifically to an invariant core motif C/A GGA A/T in the middle of 10 bp of DNA (Wasylyk et al., 1993), suggesting that their specificity is conferred by sequences flanking the GGA core. Ets members can cooperate with other transcription factors. For example, Ets1 can cooperate with c-Fos and c-Jun to increase the transcriptional activity of the polyoma virus enhancer (Wasyly et al., 1990). This enhancer is used in the MAP kinase studies presented in Chapter 5. Other potentially important interactions have been found between Ets-2 and c-Fos/c-Jun (Basuyaux et al., 1997), Ets-1 and Pit-1 (Bradford et al., 1995), Elf1 and AP-1 (Wasylyk et al., 1990; Gottschalk et al., 1993; Wang et al., 1994), Elf1 and the retinoblastoma protein (Wang et al., 1993) and Elk-1 and the serum response factor (Hipskind et al., 1991).

Elf1 is a lymphoid/myeloid-specific Ets family member which has a DNA binding domain that is nearly identical to that of E74, the ecdysone-inducible *Drosophila* transcription factor required for metamorphosis (Thompson et al., 1992). Elf1 is involved in the positive regulation of several T-cell specific genes, including

IL-2 (Thompson et al., 1992), IL2Rα (John et al., 1995, 1996), and GM-CSF (Wang et al., 1994). Elf1 has also been found to be required for repression of the constitutive expression of the IL-2Rα gene in non-activated T lymphocytes (Lecine et al., 1996).

3.1.3. NFAT transcription factor

Nuclear factor of activated T cells (NFAT) is a family of proteins related to the NF-kB family. Initially identified as a T cell-specific transcription factor that bound to the IL-2 promoter upon antigen stimulation, NFAT activity has now been demonstrated in a variety of cell types (Rao et al., 1997). The NFAT family contains at least four members: NFATp, NFATc, NFAT3 and NFAT4 (Hoey et al., 1995; Masuda et al., 1995). Each of these NFAT proteins contains a series of serine/proline-rich repeats at N terminus and a downstream Rel homology domain (RHD). The RHD is essential for DNA binding and dimerization with basic region/leucine zipper proteins such as the AP-1 family members Jun and Fos (Rooney et al., 1995). In resting T cells, NFAT proteins are present in the cytoplasm as phosphorylated forms. When T cells are activated, the level of the calcium-activated phosphatase calcineurin is elevated, which subsequently results in dephosphorylation of NFAT (Clipstone and Crabtree, 1992; Beals et al., 1997). The dephosphorylated NFAT is quickly translocated into the nucleus where, in synergy with AP-1, Maf, and NIP45 proteins, it transactivates a variety of cytokine genes and other activation genes including IL-2, IL-4, and GM-CSF (Cockerill et al., 1993; Rooney et al., 1995; Hodge et al., 1996). All NFAT members can bind to and transactivate the same set of NFAT sites derived from the IL-2 and IL-4 promoters, although NFATp and NFATc account for the majority of the binding activity (Rao et al., 1997; Timmerman et al., 1996). However,

the expression pattern and tissue distribution of each of the NFAT proteins suggests that they subserve different functions. Three of the four NFAT members (NFATp, NFATc, and NFAT4) are expressed in lymphoid organs and NFATp and NFATc are constitutively expressed at low levels. Only NFATc is up-regulated upon stimulation through the T cell receptor (Hoey et al., 1995; Masuda et al., 1995). Additional evidence for distinct functions of NFAT proteins comes from the analysis of NFATp- and NFATc-deficient mice. NFATp-deficient mice have lymphoid hyperplasia with hyperproliferation and elevated levels of IL-4 and IgE. NFATc deficiency causes cardiac valvular defects and defects in IL-4-driven responses (Hodge et al., 1996; Xanthoudakis et al., 1996; Ranger et al., 1998).

3.1.4. AP-1 transcription factor

AP-1 transcription factors are immediate early response genes involved in a diverse set of transcriptional regulatory processes (Angel and Karin, 1991). The AP-1 complex consists of a heterodimer of a Fos family member and a Jun family member. This complex binds the consensus DNA sequence (TGAGTCA) sites found in a variety of promoters (Ransone and Verma, 1990). The Fos family contains four proteins (c-Fos, FosB, Fra-1, and Fra-2) (Cohen and Curran, 1988; Zerial et al., 1989; Nishina et al., 1990), while the Jun family is composed of three members (c-Jun, JunB, and JunD) (Bohmann et al., 1987; Ryder et al., 1989). Fos and Jun are members of the bZIP (basic zipper) family of sequence-specific dimeric DNA-binding proteins (Baxevanis and Vinson, 1993), which have a DNA-binding domain consisting of a leucine zipper that is critical for the dimerization of the bZIP proteins (Landschulz et al., 1988) and a basic region that is critical for sequence-specific DNA

binding (Shuman et al., 1990). Although Jun proteins form very stable heterodimers with Fos- and ATF-family members, they can also homodimerize among themselves (Ziff, 1990). Jun-Jun and Jun-Fos dimers preferentially bind to the TPA-responsive element (TRE; this element has the base sequence TGAG/CTCA), whereas Jun-ATF dimers or ATF homodimers prefer to bind to the cAMP-responsive element (CRE; this element has the base sequence TGACGTCA) (Hai and Curran, 1991). Both elements are palindromic and contain the same AP-1 half-site.

3.1.5. **Reporter gene systems**

Reporter genes provide easy and efficient methods for the indirect measurement of relative rates of transcription. Utilizing common DNA cloning methods, a putative regulatory region can be coupled to the coding sequence of a reporter gene such that expression of the reporter protein product varies according to the regulatory potential of the DNA tested. The assays for reporter enzymes have the advantage of high sensitivity with low background and, although an indirect measure, the amount of protein product is usually directly proportional to the level of transcriptional activation. The direct measurement of the level of specific mRNAs for endogenous genes can be influenced by RNA stability changes and assays for mRNA are also more labor-intensive and difficult to quantify.

The use of reporter systems allows the detailed mapping of specific enhancer and silencer regulatory elements in transcriptional control regions. The region of DNA containing the full potential for transcriptional activation can be sequentially deleted and tested in the reporter system to identify the types of elements present. Loss or enhanced activation of reporter activity is easily measured and indicates the

position of active elements within the DNA. These individual elements can be further characterized by inserting one or multiple copies of the element immediately upstream of a heterologous basal promoter in the reporter construct. Weak constitutive promoters, such as the minimally active fragment of the Herpes Simplex Virus thymidine kinase promoter, are usually used for this purpose (Wegner et al., 1994). An enhancer region, for example, should confer a stimulus-specific or cell-specific transcriptional response on the basal promoter with the DNA fragment inserted in either orientation. On the other hand, a silencer element would result in an inhibition of reporter expression when used with a strong promoter such as a viral promoter (Wegner et al., 1994).

Reporter genes are again useful in the analysis of the transcription factors that bind to DNA at specific *cis* acting elements and control the function of these elements. Mutations can be made in specific regulatory elements within enhancer/promoter regions which prevent protein binding and the effect on reporter expression tested. In addition, transcription factors can be over-expressed or their expression inhibited with antisense methods to determine the role of individual factors in the activity of a specific element. Finally, reporter systems can provide a useful endpoint measurement for receptor structure-function studies and the delineation of components within signal transduction pathways.

Numerous reporter systems have been developed to accurately measure transcription rates. The two most commonly used systems are based on the chloramphenicol acetyltransferase (CAT) gene and the firefly luciferase gene (Gorman et al., 1982). Although both assay systems can provide an accurate and linear quantitation of transcription rates, they differ in sensitivity, ease of

performance, quantifiable range and the stability of proteins within the cells. The main advantages of the luciferase system are the nonisotopic assay of bioluminescence, the high degree of sensitivity, the absence of endogenous enzyme activity in eukaryotic cells, the wide linear range of quantitation and the ease and speed of assay. The production of advanced instrumentation such as luminometers that read 96 well plates allows an even more rapid and extensive collection of data.

In the present work, the luciferase reporter gene system was used to investigate the transcriptional regulation of the IL-5 gene. The pXPG luciferase reporter gene vector used in this study has been described in detail (Bert et al., 2000).

3.1.6. Stable versus transient transfection systems

In stable transfection, DNA is permanently integrated into the normal chromatin environment of the host cell. Exogenous DNA in a chromosomal environment is able to interact with the normal regulatory mechanisms of the cell, including DNA replication, nuclear matrix and nucleosomal spacing and positioning (Wolff, 1994). It is now clear that chromatin structure plays an important role in the regulation of gene expression. Since all cellular genes are packaged into ordered chromatin structures, an understanding of the mechanisms by which nucleoprotein structure influences transcriptional activation is necessary for a complete understanding of gene regulation in higher eukaryotes.

However, structural analysis of stably integrated gene promoters in mammalian cells requires the time-consuming generation of multiple stable cell lines or pools, in which the integrated genes could be subject to position effects from surrounding chromatin. Therefore, the identification and characterization of factors involved in

gene expression have been addressed primarily through the use of transient transfection assays. In this approach, exogenous plasmid DNA, usually promoter/reporter constructs and transcription factor expression vectors, is introduced into cultured cells and expressed transiently, without replication or integration into the cellular genome. These studies have resulted in an abundance of information about promoter structure and function and have allowed the characterization of transcription factor interactions that are part of various cellular regulatory pathways.

The disadvantages of the transient transfection system are that the bacterial methylation pattern of the exogenous DNA and the artificial state of the DNA outside the normal chromatin environment of the cell may affect the transcription. The appropriate genomic enhancers and/or repressors also may not function normally outside of their usual chromatin environment. In addition, the large amounts of plasmid DNA entering the cell may interfere with the coordinated assembly of the transcription apparatus on a particular template by competitively displacing critical factors which may be in low abundance.

3.1.7. Transfer of DNA into cells by electroporation

A variety of transfection methods have been utilized to deliver DNA into cells. Four of the most commonly used transfection procedures are 1) calcium phosphate precipitation (Gorman et al., 1982), 2) DEAE-dextran-mediated transfection (Danna et al., 1982), electroporation (Toneguzzo et al., 1986), and 4) liposome-mediated transfer (Felgner et al., 1987). Among these methods, electroporation is the most widely used. In this method, an electrical pulse delivered by a capacitor is believed to

transiently disturb membrane integrity creating pores through which the plasmid DNA enters the cell. Plasmid uptake has been assumed to occur by passive diffusion, although evidence has been obtained suggesting that DNA entry is a rapid electrophoretic event (Klenchin et al., 1991).

3.1.8. The eletrophoretic mobility shift assay (EMSA)

The gel mobility shift assay is a simple and sensitive method for determining interaction between proteins and DNA. This assay (Fried and Crothers, 1981; Garner and Revzin, 1981) was developed for analysing protein-DNA interactions. In this assay, the DNA fragment or synthetic oligonucleotide containing potential transcription factor binding sites is radiolabelled. DNA binding proteins are incubated with the radiolabelled probes. The protein-DNA complexes are separated from free DNA by non-denaturing polyacrylamide gel electrophoresis. Unlike footprinting techniques, that rely on the loss of a signal to determine protein-DNA interactions (negative assay), the gel mobility shift assay yields a positive signal; the appearance of a DNA fragment with an altered mobility. An unexpected property of the gel assay is its ability to detect and physically separate alternative conformational states of complexes that rapidly equilibrate in solution. However, the gel shift assay does not give a direct readout of the DNA nucleotides that the protein is recognizing. For this type of information, a higher resolution technique such as DNase I footprinting or methylation interference is necessary. A combination of these assays can often be used with considerable advantage

3.2. Results

3.2.1. Establishment of a transient transfection system in D10W cells.

A transient transfection system was established using D10W cells. Since electroporation is an efficient, highly reproducible technique, which is suitable for suspension cell lines like D10W, this technique was used for transfection. It was necessary to determine the electrical parameters for maximum electroporation efficiency. The principle of electroporation involves the use of a high voltage pulse which causes the temporary formation of pores in the cell and nuclear membranes, making them permeable to exogenous DNA. In general, the conditions for delivery of DNA into cells result in the death of about half the cells of the population, although the extent of cell death associated with maximum electroporation efficiency is variable among cell lines. A control plasmid expressing green fluorescent protein (GFP) was used to optimize electroporation conditions for D10W cells. The percentage of cells transfected at voltages ranging from 230-310 V with a capacitance of 975 μF was determined by FACS as described in Materials and Methods. As shown in Fig.3.2, at 270 V and 975 μF capacitance, about 50% of the cells were killed and 16% of the cells expressed GFP. Using these conditions, a luciferase reporter construct (-1170mIL-5Luc) carrying 1170 bases of the upstream region of the mouse IL-5 gene was transiently transfected into D10W cells. Subsequent stimulation of the cells with PMA and cAMP stimulus which had previously been shown to give optimal expression of the IL-5 gene in D10W cells (Bourke et al., 1995; Tan, 1998; Young et al., 1999) gave significant induction of luciferase expression. A time course of expression of -1170mIL-5Luc was determined and it

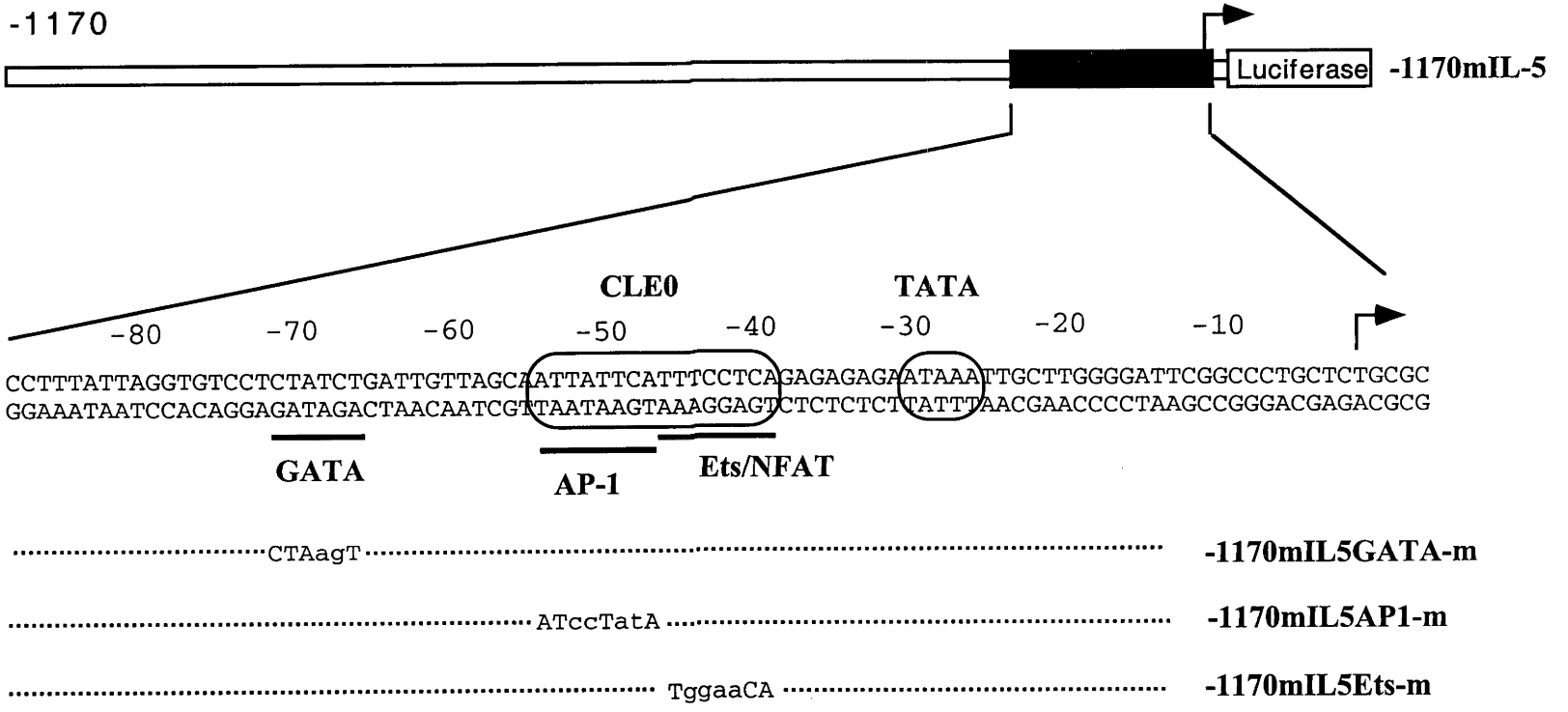


Fig. 3.1. Structure of the mouse IL-5 promoter luciferase construct showing the DNA binding sites studied in the present work. The -1170 bp mouse IL-5 promoter contains a transcriptional control region of approximately 90 bp proximal to the transcriptional initiation site. This region contains elements which are conserved among the promoters of genes expressed following T cell activation. The CLE0 element contains binding sites for AP-1 and Ets/NFAT families. Upstream of the CLE0 element, a GATA transcription factor binding element is found. Other elements include a consensus TATA box. The sequences of oligonucleotides spanning the GATA, AP-1 and Ets/NFAT sites and the mutations used to inactivate them are shown. The -1170mIL-5 is a construct driven by the 1170 bp wild-type IL-5 promoter. Mutations in the binding sites have been introduced into the -1170mIL5-GATA-m, AP-1-m and Ets-m constructs, as indicated.

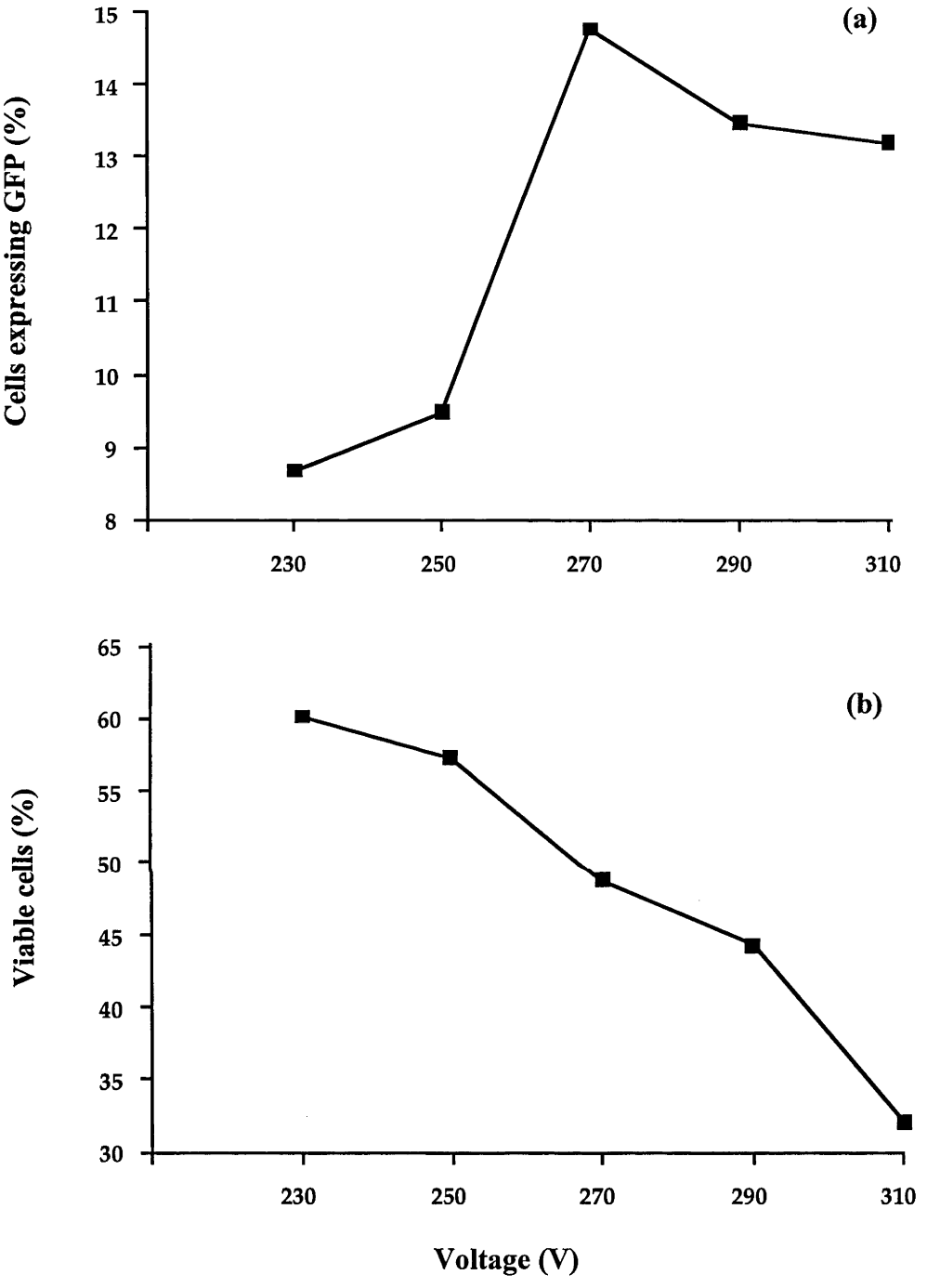


Fig. 3.2. Effect of electroporation voltage on D10W cells viability and transfection efficiency in single pulse electroporation. Five μg of GFP expression construct was transfected into D10Wcells using voltages between 230-310 V. After 20 hours recovery , transfected cells were stained with PI. The number of the cells expressing GFP (a) and cell viabilities (b) were determined by FACS analysis (see Materials and Methods).

was found that maximal induction of the IL-5 reporter construct was achieved after 9-12 hours stimulation (Fig.3.3).

3.2.2. Induction of the mouse IL-5 reporter gene by PMA and cAMP in D10W cells

Using optimal conditions for electroporation and stimulation, the -1170mIL-5Luc construct was transiently transfected into D10W cells. Transfected cells were treated with PMA, cAMP or both. PMA alone gave little stimulation whereas cAMP alone gave 10-fold induction. The combination of PMA and cAMP gave 36-fold induction (Fig.3.4). There was very low expression of the reporter construct in the absence of stimulation. The synergism observed between PMA and cAMP agreed well with previous findings using stable transfection assays in D10W cells and the degree of induction was sufficient to make this a good system for transactivation experiments.

3.2.3. Transactivation with expression constructs

Previous studies from this laboratory using stable transfection into the mouse Th2 clone D10W (a derivative of D10.G4.1) have indicated that the proximal promoter region to -88 controls the inducible expression of the mouse IL-5 gene in Th2 lymphocytes (Tan, 1998; Young et al., 1999). This region carries putative binding sites for Ets/NFAT (-41 to -47), AP-1 (-48 to -54) and GATA (-67 to -72) (Fig.3.1). It was previously shown by mutational studies that each of these sites is required for inducible expression of IL-5 reporter gene constructs stably incorporated into the genome.

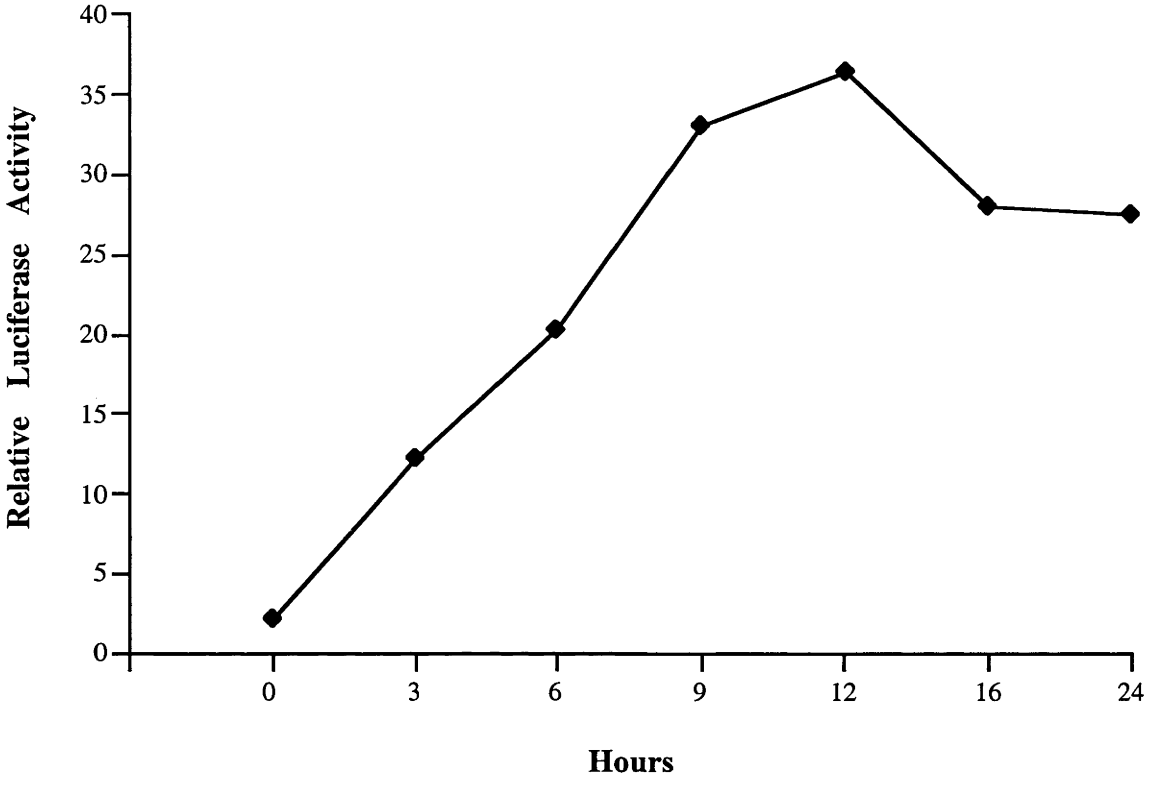


Fig. 3.3. Time course of mouse IL-5 luciferase reporter gene expression in D10W cells in response to PMA and cAMP stimulation. D10W cells were transiently transfected with the -1170mIL-5Luc construct. Transfected cells were treated with PMA and cAMP and luciferase activity was measured between 0-24 hours (see Materials and Methods).

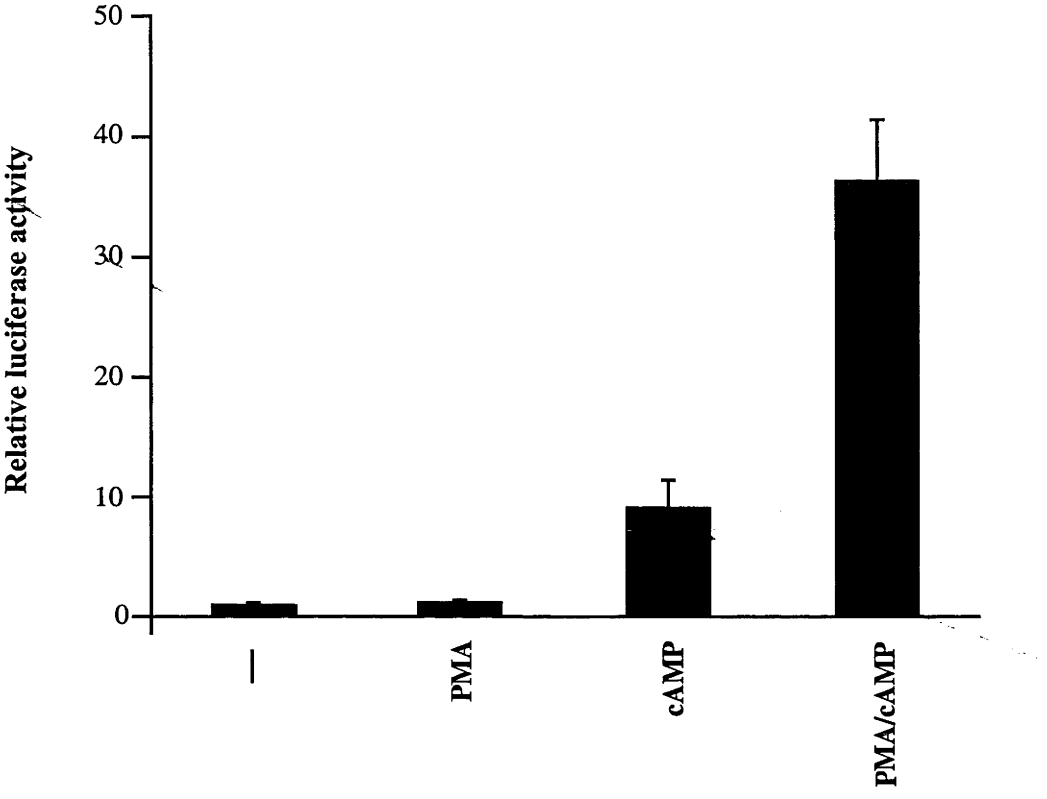


Fig. 3.4 Expression of mouse IL-5 promoter in D10W cells. The -1170mIL-5Luc construct was transiently transfected into D10W cells. After 20 hours recovery, transfected cells were stimulated with PMA or cAMP, and both PMA and cAMP for 9 hours and uciferase activity measured (see Materials and Methods). The results represent the average of at least three independent experiments

Transactivation experiments in transient expression assays using expression constructs encoding relevant transcription factors were therefore carried out to identify those that could stimulate expression of the IL-5 gene. The most likely factors to be involved in binding to the respective sites were AP-1 (AP-1 site), Ets1, Elf1, NFATp or NFATc (Ets/NFAT site) and GATA-3 (GATA site). Since a role for GATA-4 in the expression of the human IL-5 gene has been reported (Yamagata et al., 1995,1997), GATA-4 was also tested. Expression constructs for these transcription factors were transiently cotransfected together with the -1170mIL-5Luc reporter construct into D10W cells and the transcriptional activity of the IL-5 promoter was measured with and without stimulation.

As shown in Fig.3.5a, in the absence of stimulation, dramatic transactivation of approximately 400-fold over normal unstimulated levels of expression was achieved with an AP-1 expression construct. Smaller increases in expression of up to 4-fold were obtained with expression constructs for GATA-3 and GATA-4. There was no significant transactivation of the IL-5 promoter by Elf1. Ets1. NFATp and NFATc gave small increases of up to 2-fold (Fig.3.5a). The effect of stimulation by PMA, cAMP or both on the level of transactivation was also tested. In each case, PMA stimulation resulted only in a minor increase in transactivation. In contrast, cAMP was considerably more effective and strong synergistic stimulation was observed when PMA and cAMP were used together (Fig3.6a-d). In the transactivation experiments, responses to stimulation by PMA, cAMP or both agents were similar to the responses observed with the -1170mIL-5Luc plasmid alone (Fig.3.6a-d), although significantly higher values of IL-5 expression were obtained by transactivation. For AP-1, the values after transactivation and stimulation with PMA and cAMP were

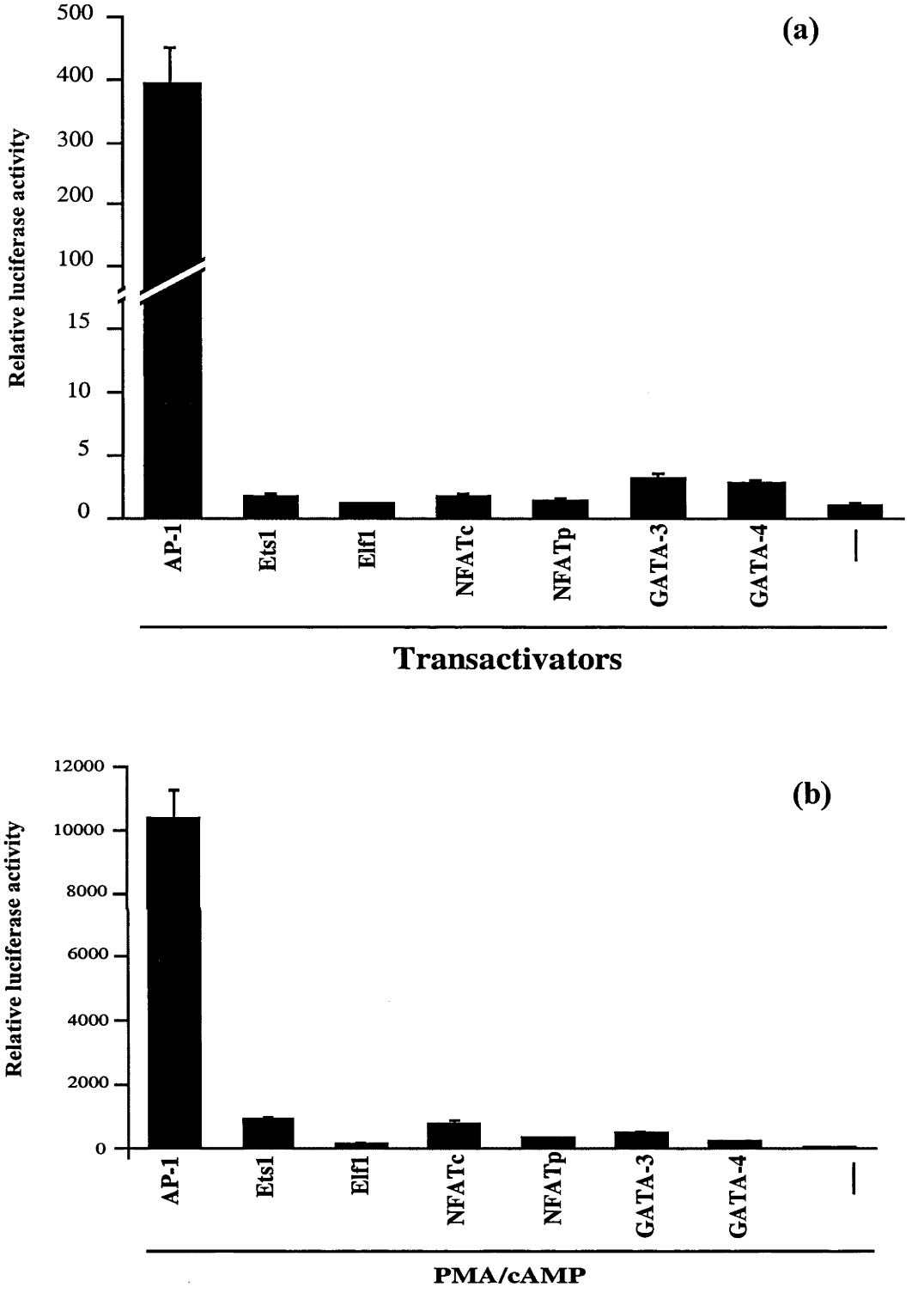


Fig. 3.5 Transactivation of expression of IL-5 promoter using expression constructs for relevant transcription factors. The -1170mIL-5Luc construct was cotransfected with various expression vectors for relevant transcription factors into D10W cells. Luciferase activity was measured after 9 hours (see Materials and Methods). (a) unstimulated; (b) stimulated with PMA and cAMP. The results represent the average of at least three independent experiments.

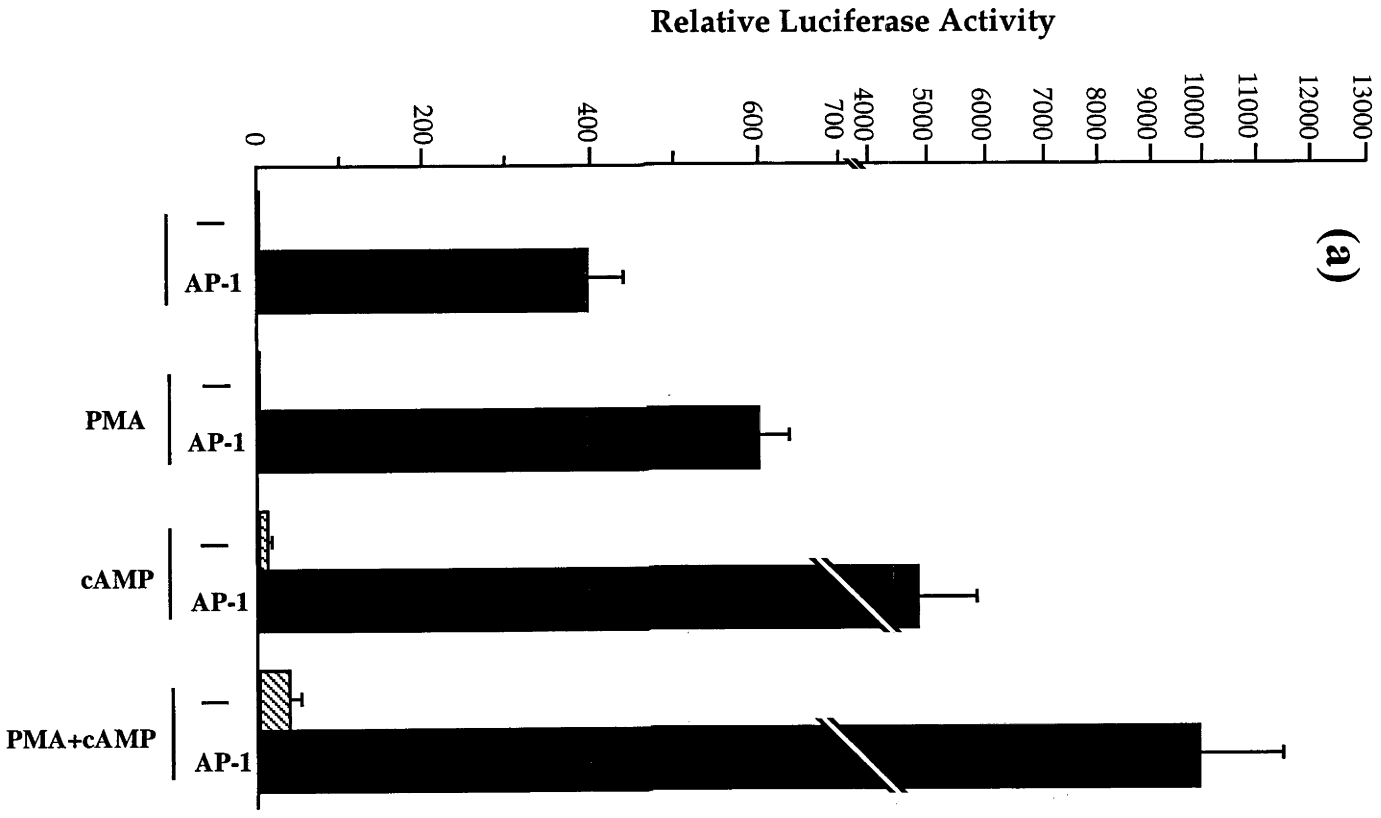
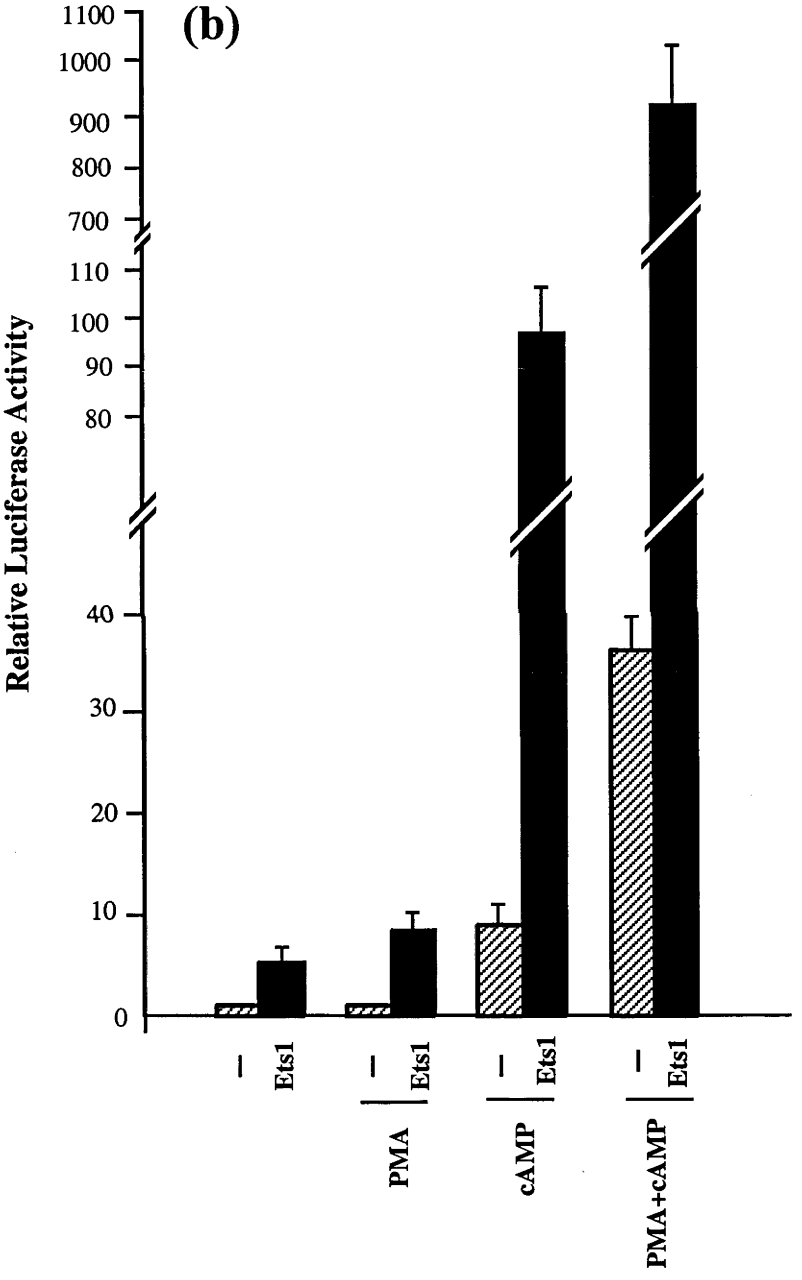
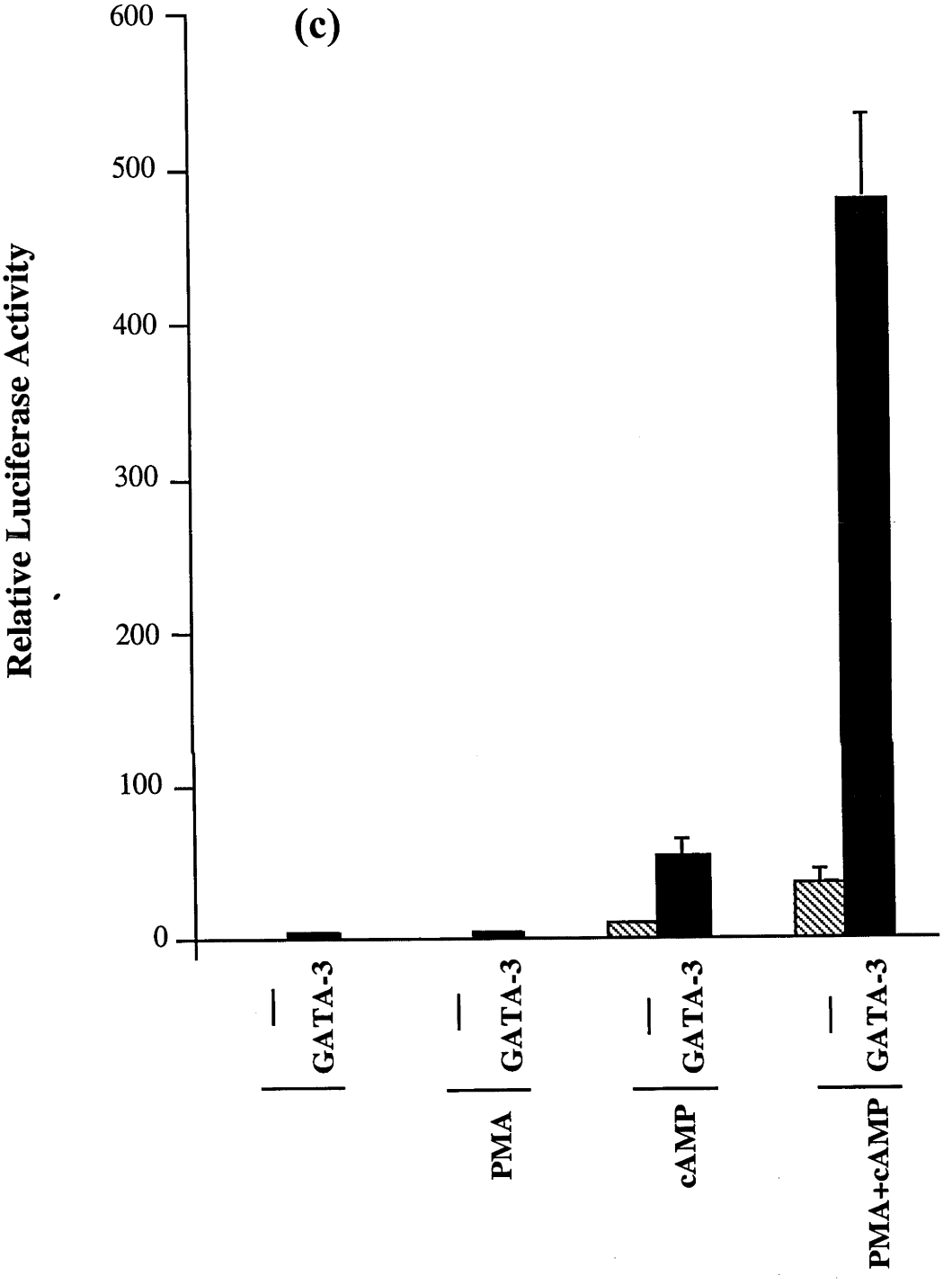
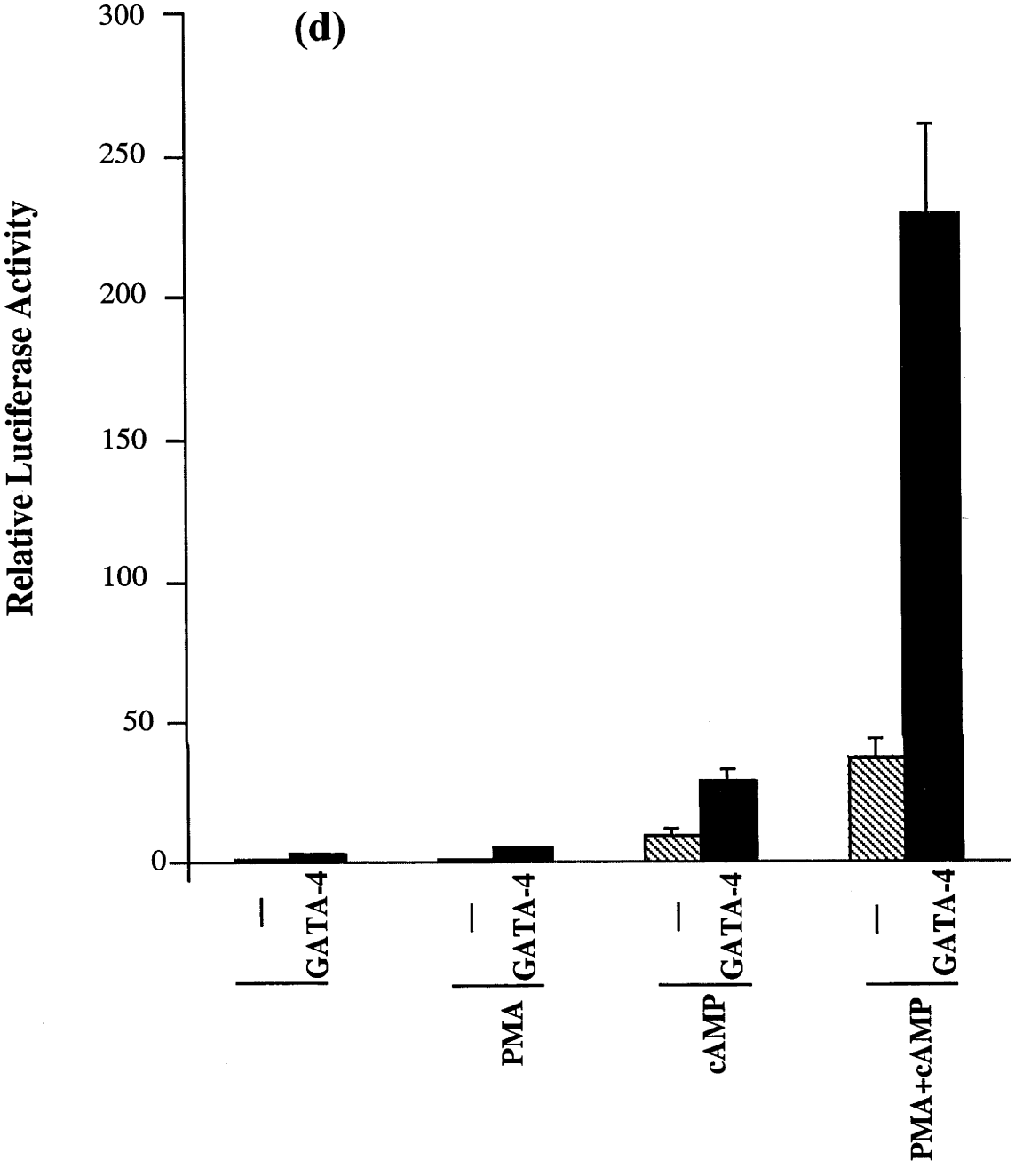


Fig. 3.6 Effect of stimulation by PMA and cAMP on transactivation of the IL-5 promoter in D10W cells. The -1170mIL-5Luc construct was cotransfected with different expression plasmids into D10W cells. Transfected cells were stimulated with PMA or cAMP and both PMA and cAMP and luciferase activity was measured (see Materials and Methods). (a) transactivation by AP-1; (b) Ets1; (c) GATA-3; (d) GATA-4. The results represent the average of at least three independent experiments.


(b)
Relative Luciferase Activity
1100
1000
900
800
700
110
100
90
80
40
30
20
10
0
–
Ets1
–
Ets1
PMA
–
Ets1
cAMP
–
Ets1
PMA+cAMP


(c)
Relative Luciferase Activity
600
500
400
300
200
100
0
—
GATA-3
—
PMA
GATA-3
—
cAMP
GATA-3
—
PMA+cAMP
GATA-3


(d)
Relative Luciferase Activity
300
250
200
150
100
50
0
—
GATA-4
—
PMA
GATA-4
—
cAMP
GATA-4
—
PMA+cAMP
GATA-4

250-fold higher than with -1170mIL-5Luc alone (Fig.3.5b). Transactivation by Elf1, NFATp and NFATc was also tested following stimulation with PMA and cAMP. Like Ets1, these transcription factors could also potentially bind at the putative Ets/NFAT site. Elf1 and NFATp gave lower levels of transactivation than Ets1 and NFATc(Fig.3.5b). In these experiments Ets1 and NFATc gave similar levels of transactivation. However, the involvement of Ets1 at the Ets/NFAT site is supported by its unique ability to synergize with AP-1 (see below).

In view of the high levels of transactivation of the -1170mIL-5Luc construct achieved with AP-1 (c-Fos/c-Jun), experiments were carried out to determine the level of transactivation by c-Fos and c-Jun alone. Expression constructs were transfected together with -1170mIL-5Luc into D10W cells and the cells stimulated with PMA and cAMP. Transactivation by c-Jun was much more effective than by c-Fos but transactivation by a combination of the two was by far the most effective (Fig.3.7a). The low level of transactivation by c-Fos is consistent with the inability of c-Fos to form transcriptionally active dimers. The moderate activity of c-Jun in comparison to AP-1 indicates that c-Jun homodimers are less active than c-Fos/c-Jun heterodimers in stimulating transcription from the IL-5 promoter.

3.2.4. Repression of the transcriptional activity of the IL-5 promoter by dominant negative AP-1

Since the transactivation experiments showed a very strong stimulation by AP-1, experiments were carried out to see if normal induction of IL-5 expression could be inhibited by dominant negative AP-1 expression constructs. Dominant negative c-Fos and c-Jun have been made by fusing an acidic amphipathic extension to the N-

terminus of the c-Jun and c-Fos leucine zipper domains. The acidic extension of c-Jun and c-Fos interacts with the basic region of wild type c-Jun and c-Fos forming a coiled-coil extension of the leucine zipper and thus prevents the basic region of wild-type c-Jun and c-Fos binding to DNA (Olive et al., 1997)

To test for repression of IL-5 expression, dominant negative c-Fos and c-Jun expression plasmid constructs were cotransfected together with -1170mIL-5Luc into D10W cells and IL-5 expression induced with PMA and cAMP. Dose-dependent repression of the IL-5 promoter activity by a combination of dominant negative c-Fos and c-Jun was observed which was very significant at higher levels of transfected DNA (Fig.3.7b). The dominant negative c-Fos construct alone also significantly inhibited the transcriptional activity of the mouse IL-5 promoter consistent with the other evidence obtained of strong involvement of AP-1 in IL-5 gene expression (Fig.3.7c).

3.2.5. Ets1 and AP-1 synergistically transactivate the IL-5 promoter

Since the Ets and AP-1 binding sites are adjacent in the proximal region of the mouse IL-5 promoter and Ets1 and AP-1 have been reported to form a complex in T lymphocytes (Bassuk et al., 1995), it was of interest to determine if AP-1 could synergize with Ets1 or any of the other transcription factors potentially able to bind to the Ets site. Therefore an expression construct for AP-1 was transiently cotransfected in combination with expression constructs for the other relevant transcription factors into D10W cells. Without stimulation, AP-1 and Ets1 gave strong synergistic transactivation of the -1170mIL-5Luc reporter construct elevating expression levels to 10 times the additive stimulation of AP-1 and Ets1 alone. None of the other

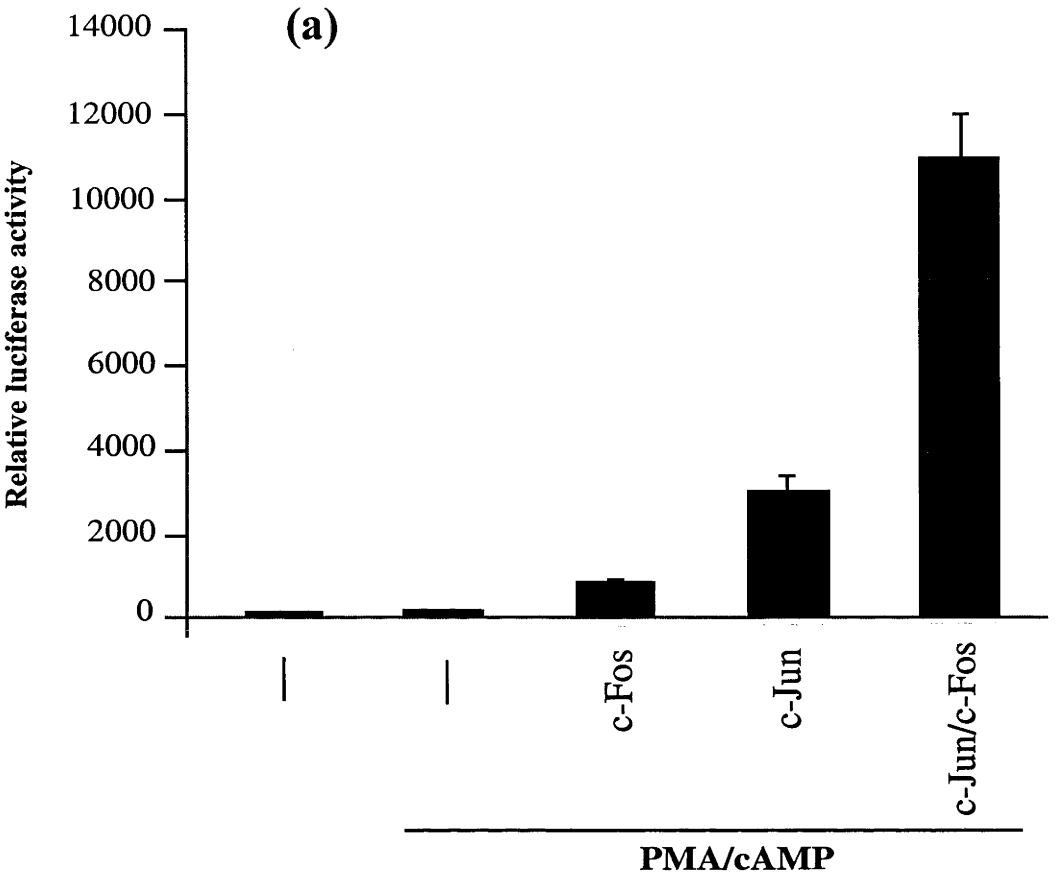
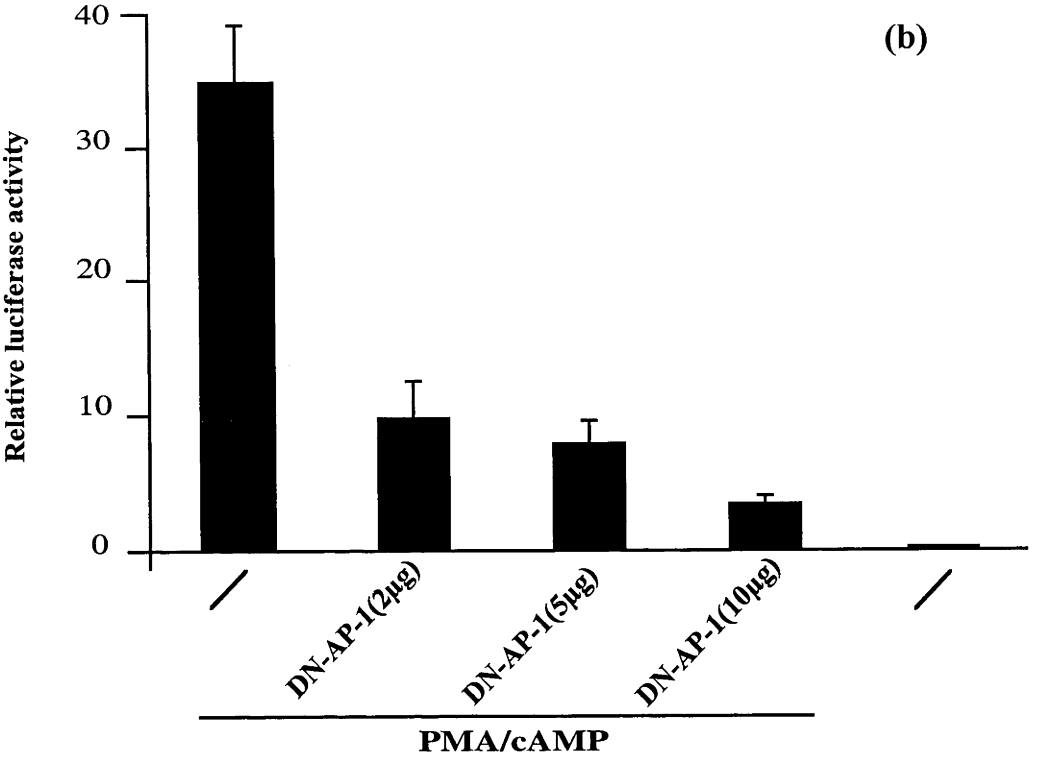
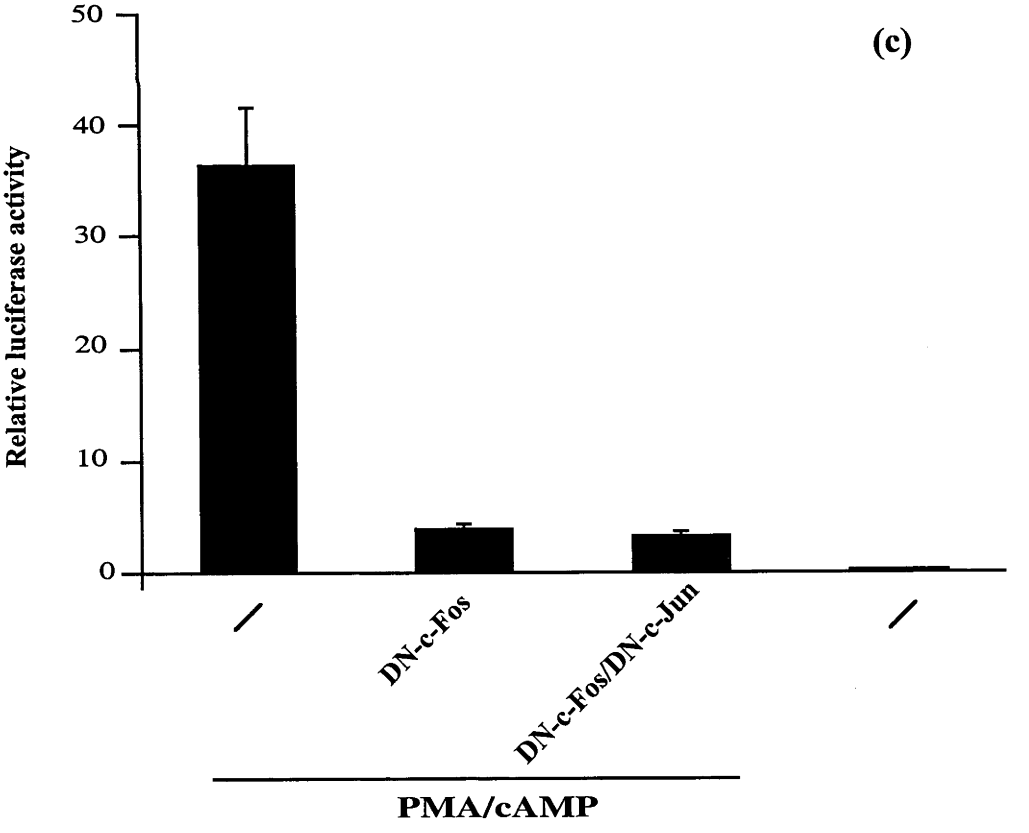


Fig. 3.7 Role of c-Fos and c-Jun in IL-5 promoter expression. D10W cells were cotransfected with the -1170mIL-5Luc construct and expression plasmids or dominant negative (DN) constructs for either c-Fos or c-Jun or both. Luciferase activity was measured after stimulation with PMA and cAMP (see Materials and Methods). (a) transactivation by c-Fos and c-Jun; (b) effect of dominant negative-AP-1 at different levels; (c) effect of dominant negative c-Fos alone. The results represent the average of at least three independent experiments.


(b)
Relative luciferase activity
40
30
20
10
0
DN-AP-1(2μg)
DN-AP-1(5μg)
DN-AP-1(10μg)
PMA/cAMP


(c)
Relative luciferase activity
50
40
30
20
10
0
DN-c-Fos
DN-c-Fos/DN-c-Jun
PMA/cAMP

transcription factors tested, including GATA-3 and GATA-4 synergized strongly with AP-1 (Fig.3.8). The demonstrated synergy between AP-1 and Ets1 further supports the involvement of Ets1 in regulating IL-5 expression at the Ets/NFAT site.

3.2.6. Mutation of the putative AP-1, Ets/NFAT and GATA binding sites in the proximal promoter region

To investigate whether the transcriptional activity of the IL-5 gene induced by PMA and cAMP in transient transfection assays was dependent on the three transcription factor binding sites in the proximal promoter region (Fig.3.1), individual mutations in the AP-1, Ets/NFAT and GATA sites were generated in the –1170mIL-5Luc construct. Transient transfection experiments showed that each mutation resulted in very little change in the basal level of expression. However, mutation of each of the three transcription factor binding sites resulted in almost complete abolition of IL-5 expression in response to PMA and cAMP treatment (Fig.3.9a) indicating that the AP-1, Ets/NFAT and GATA binding sites are all required for the promoter activity of the IL-5 gene in transient transfection assays. These findings are in complete agreement with the results obtained from stable transfection assays with the IL-5 gene in a chromatin environment (Tan, 1998; Young et al., 1999)

To determine if the transactivation of IL-5 expression by AP-1, Ets1 and GATA-3 was also dependent on the AP-1, Ets/NFAT and GATA sites in the proximal promoter region, the transactivation experiments were therefore repeated using derivatives of the –1170mIL-5Luc construct carrying mutations at the AP-1, Ets/NFAT or GATA sites (Fig.3.1). In each case, the transactivations observed with AP-1, Ets1 or GATA-3 were reduced to 1% or less by mutation of the relevant

transcription factor binding sites (Fig.3.9b). In addition, each transactivation was also reduced to a low level by mutating either of the other two binding sites. This indicates that the normal mechanism of gene induction is involved in the transactivations observed and gives further indication that each of the three binding sites is obligatorily required for expression of the IL-5 gene.

3.2.7. Transactivation experiments with the proximal promoter region

The proximal promoter region to -88 carries the AP-1, Ets/NFAT and GATA sites (Fig.3.1). Previous work in this laboratory has shown that this region is important in the induction of the promoter and in its tissue-specific expression in Th2 lymphocytes (Tan, 1998; Young et al., 1999) However, the stably transfected proximal promoter construct shows differences in its induction and repression characteristics to the construct which carries the upstream region to -1170 (Tan, 1998; Young et al., 1999). For example, stably transfected -88mIL-5CAT is fully induced by cAMP alone and not repressible by dexamethasone (Tan, 1998; Young et al., 1999). This indicates the involvement of one or more additional transcription factors binding further upstream in normal gene induction and repression. In addition, there have been a number of reports from other laboratories of the involvement of upstream regulatory element in mouse IL-5 expression. These include: mPRE1-IL5 (-90/-79, Schwenger et al., 1998); IL-5P (-117/-92, Tsuruta et al., 1995), IL-5PRE (-224/-88, Stranick et al., 1995) and mPRE2- IL5 (-470/-459, Schwenger et al., 1998). Some negative elements have also been reported including: NRE-II (-300/-261, Stranick et al., 1995), NRE-I (-431/-392, Stranick et al., 1995) and CTF/NF1 (-940/-928, Bourke et al., 1995).

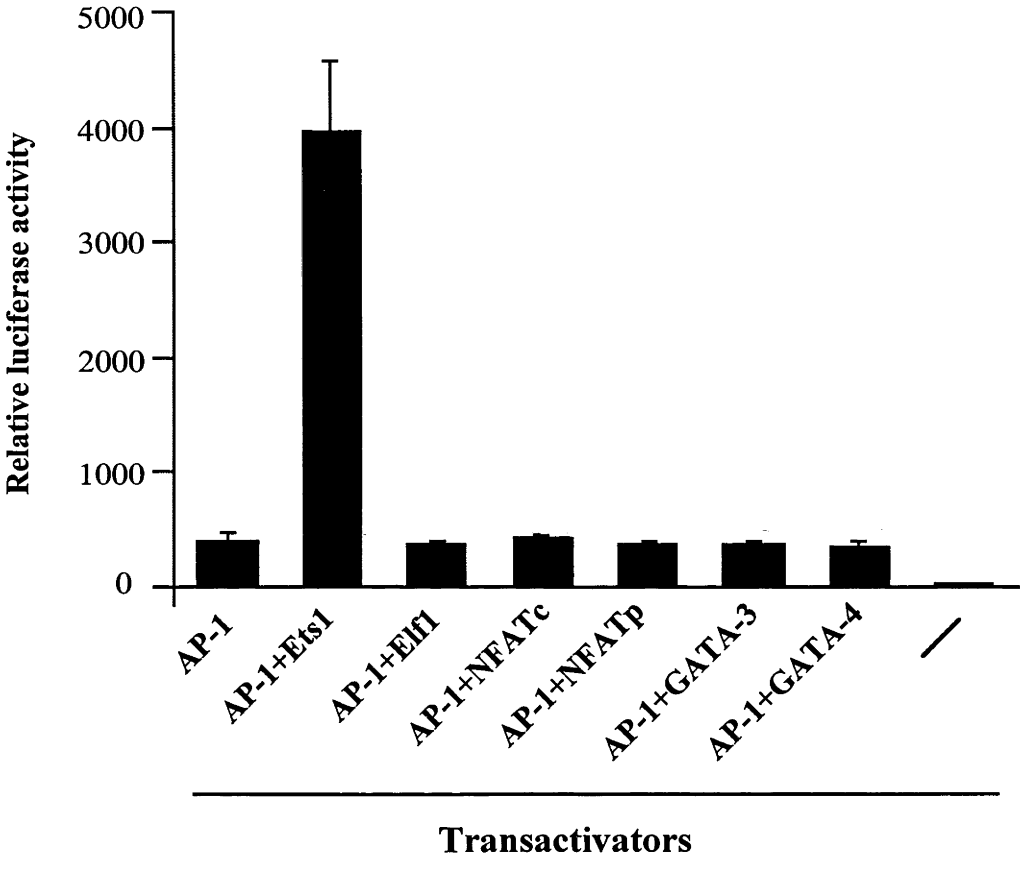


Fig. 3.8 Synergistic transactivation of IL-5 promoter by AP-1 and Ets1. The -1170mIL-5Luc was cotransfected with the combinations of various expression plasmids into D10W cells. Luciferase activity was measured(see Materials and Methods). Results represent the average of at least three independent experiments.

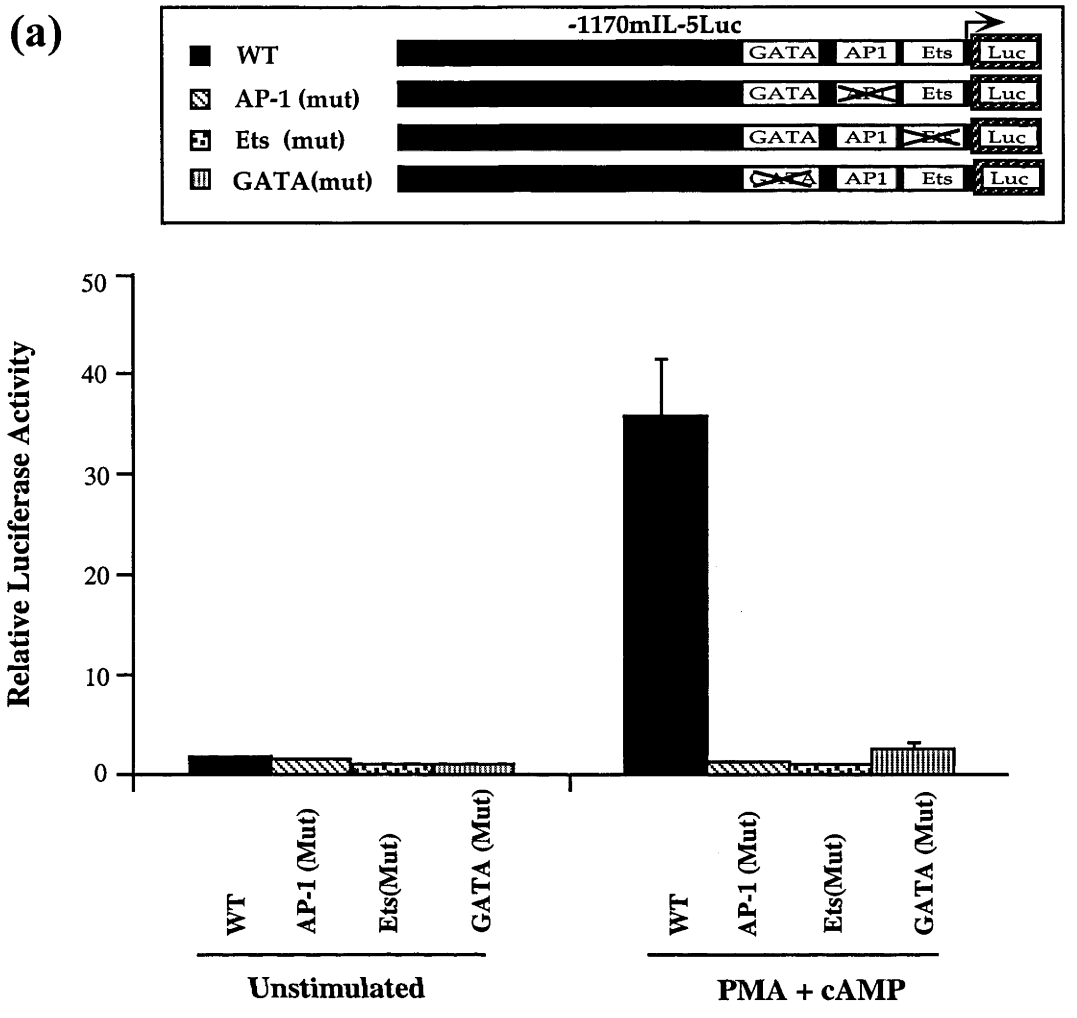
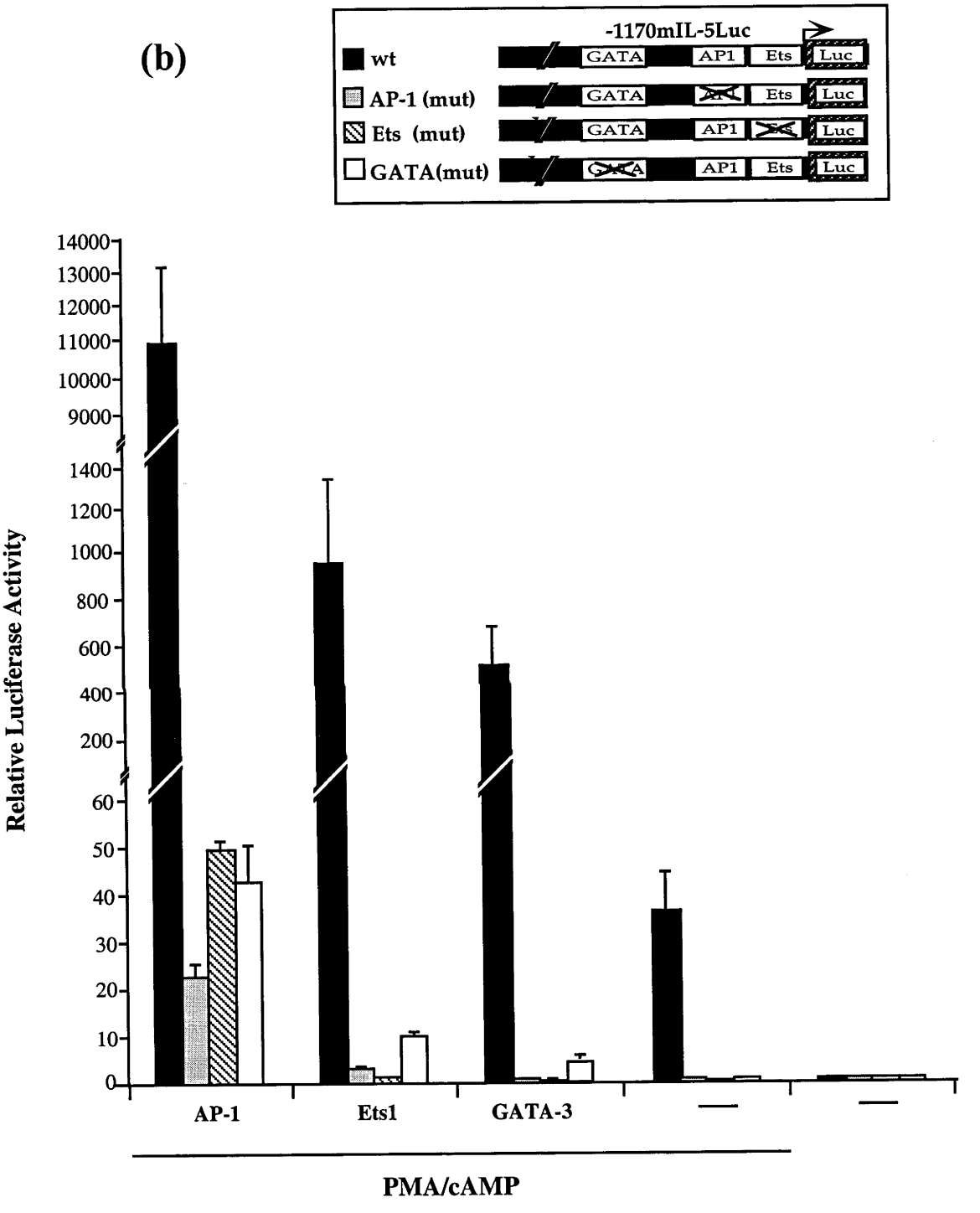


Fig. 3.9 Effect of mutation of the respective transcription factor binding sites on the expression of IL-5 promoter. D10W cells were transfected with wild-type or mutant -1170mIL-5Luc constructs as indicated. Transfected cells were either left unstimulated or stimulated with PMA and cAMP and luciferase activity was measured (see Materials and Methods). (a) wild-type and mutant -1170mIL-5Luc constructs alone; (b) -1170mIL-5Luc constructs transactivated with either AP-1, Ets1 or GATA-3. The results represent the average of at least three independent experiments. The mutations carried by the different constructs are indicated and described in detail in Fig. 3.1.


(b)
-1170mIL-5Luc
wt
AP-1 (mut)
Ets (mut)
GATA(mut)
GATA
AP1
Ets
Luc
Relative Luciferase Activity
14000
13000
12000
11000
10000
9000
1400
1200
1000
800
600
400
200
60
50
40
30
20
10
0
AP-1
Ets1
GATA-3
PMA/cAMP

In the present work, a proximal promoter construct (-88mIL-5Luc) was used to determine whether the induction by PMA and cAMP stimulation and the transactivation observed with the -1170mIL-5luc construct were also observed with the -88mIl5Luc construct and therefore not dependent on transcription factor binding sites further upstream.

The pattern of induction of -88mIL-5Luc reporter gene construct by stimulation with PMA, cAMP or both was very similar to that achieved with -1170mIL-5Luc construct (Fig.3.10). Dramatic transactivation by AP-1 (430-fold above unstimulated levels was also observed with the -88mIL-5Luc construct in the absence of stimulation (Fig.3.11a). Transactivation by the other expression constructs was much less, the strongest being with Ets1 (3-fold). When the cells were stimulated with PMA and cAMP, AP-1 transactivation gave levels over 5000-fold above those with –88mIL5Luc alone (Fig.3.11b). Transactivation by the other expression constructs gave much less stimulation than that achieved with AP-1. Moderate synergy was observed between AP-1 and NFATp, NFATc (Fig.3.11c). The highest synergy was between AP-1 and Ets1 (Fig.3.11c) although the level of synergy was much reduced compared with the -1170mIL-5Luc construct. It is clear from these results that the high levels of activation of the IL-5 gene achieved by transactivation with AP-1 are also seen with the –88mIL-5Luc construct.

3.2.8. Binding of recombinant AP-1 and Ets1 to the IL-5 promoter

Recombinant AP-1 (c-Fos/c-Jun) and Ets1 were expressed in *E.coli* together with an affinity tag (6 N-terminal histidine residues) and purified by affinity chromatography. Recombinant AP-1 protein was provided by D. Tremethick

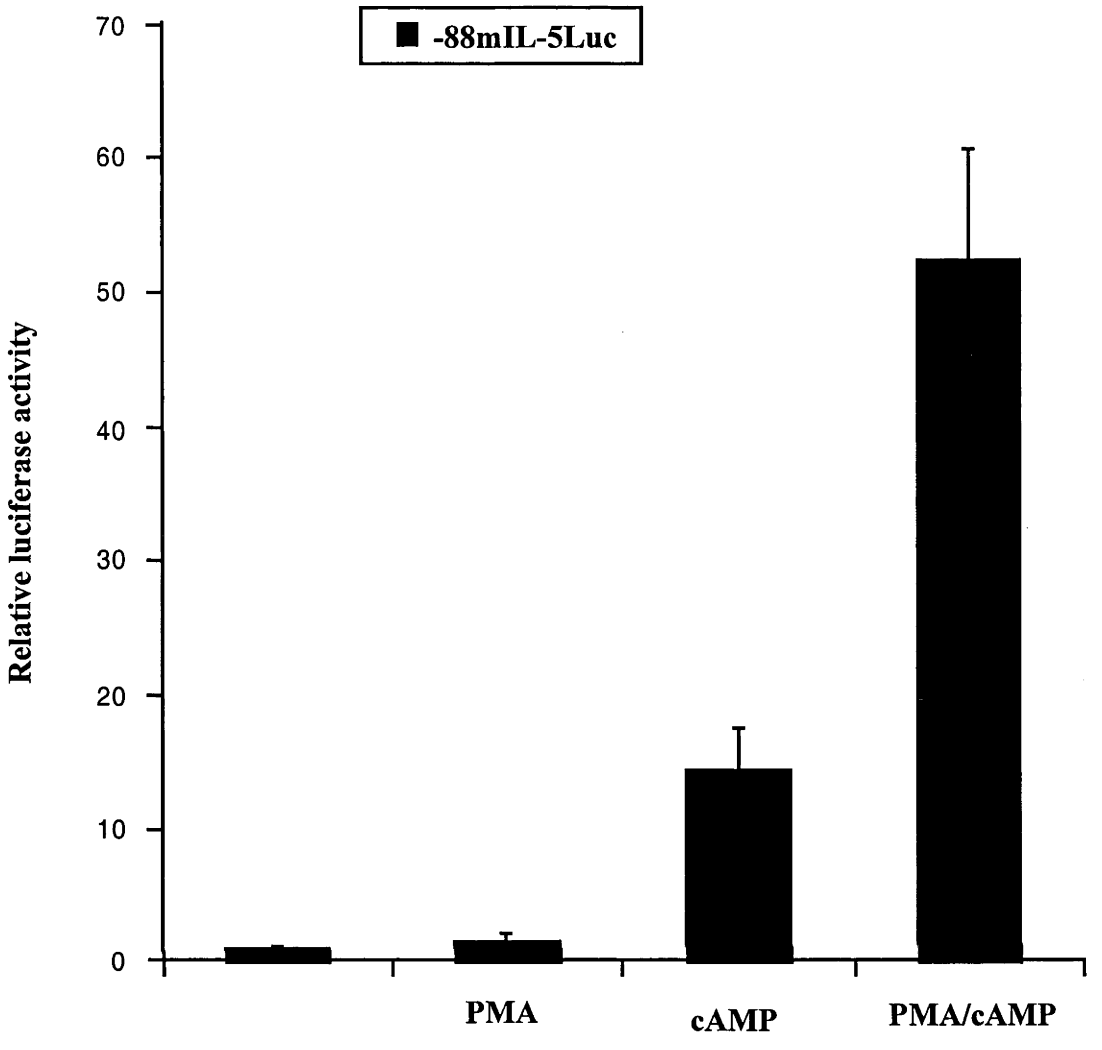


Fig. 3.10 Expression of proximal promoter of mouse IL-5 in D10W cells. The -88mIL-5Luc construct was transiently transfected into D10W cells. After 20 hours recovery, transfected cells were stimulated with PMA or cAMP, and both PMA and cAMP for 9 hours and luciferase activity was measured (see Materials and Methods). The results represent the average of at least three independent experiments.

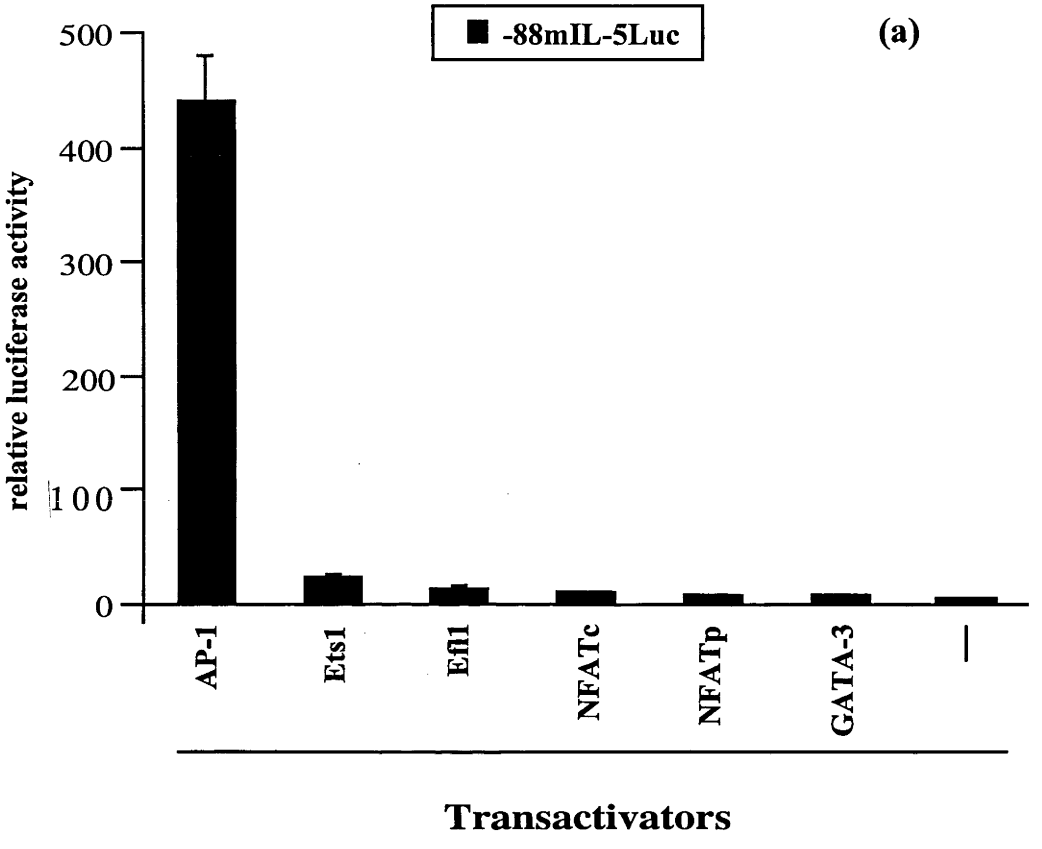
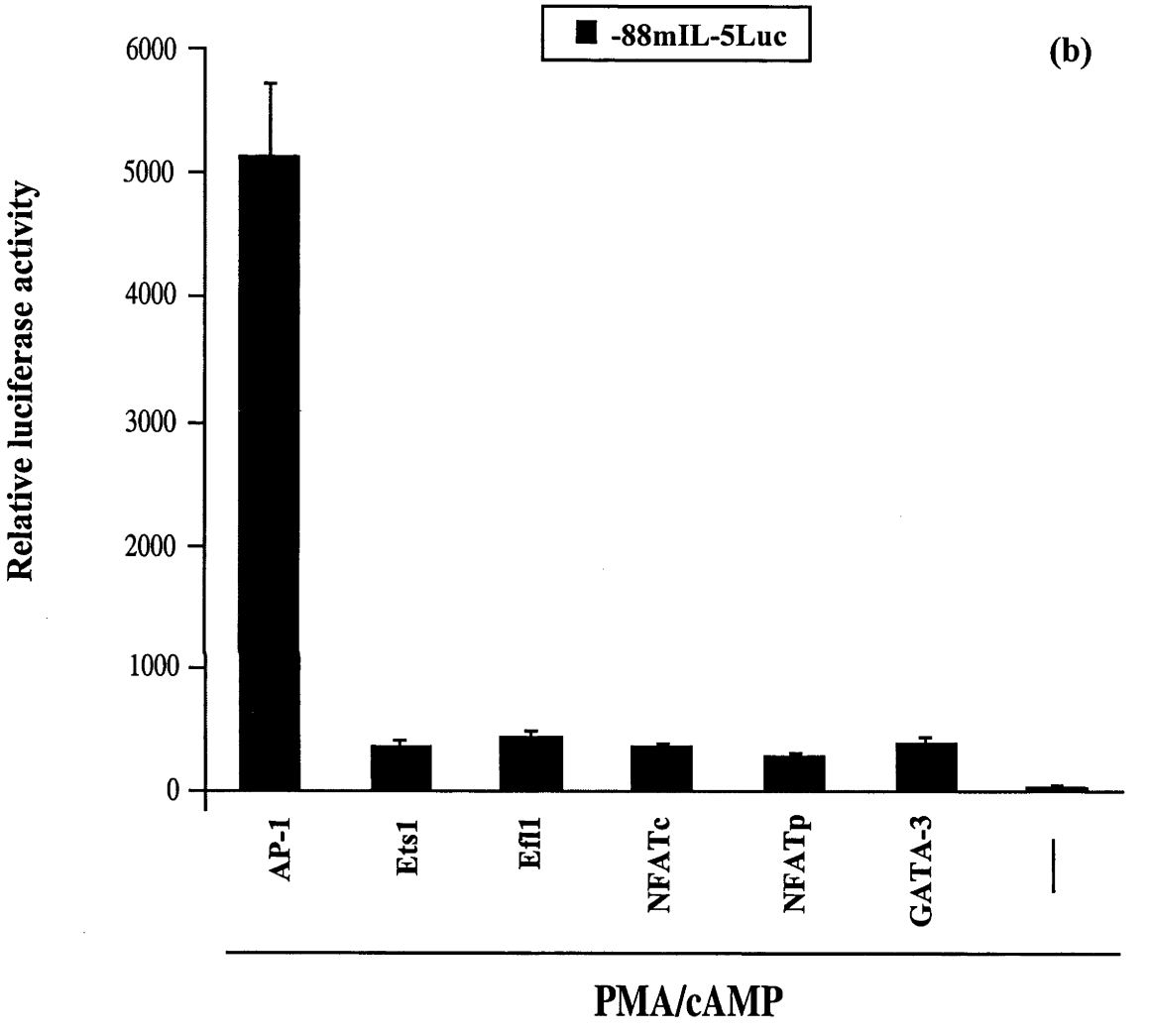
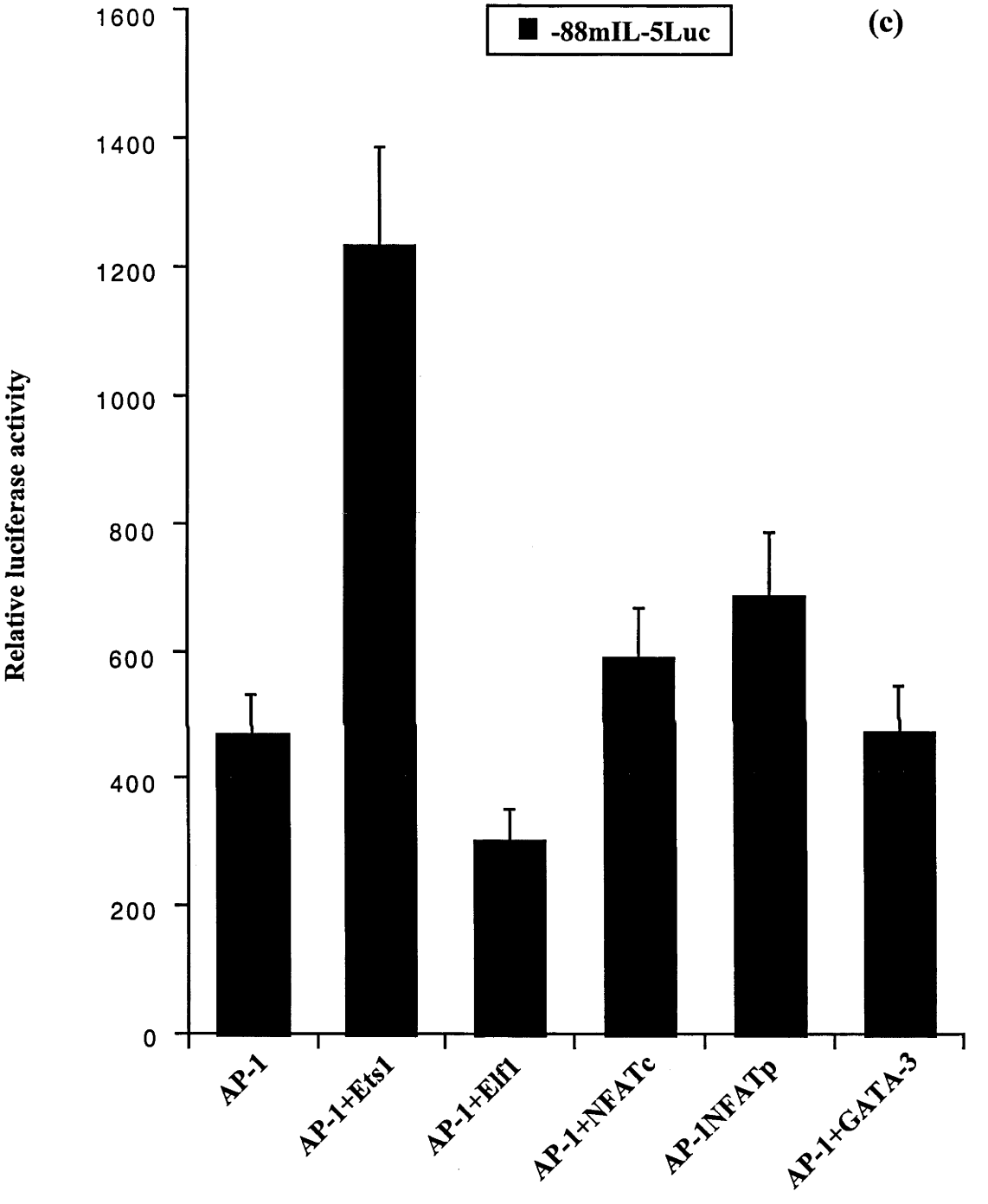


Fig. 3.11 Transactivation of the proximal promoter region of the mouse IL-5 gene promoter. The -88mIL-5Luc construct was cotransfected with various indicated expression constructs into D10W cells and luciferase activity was measured (see Materials and Methods). (a) without stimulation; (b) cells stimulated with PMA and cAMP; (c) effect of AP-1 in combination with other transcription factors. The results represent the average of at least three independent experiments.


-88mIL-5Luc
(b)
Relative luciferase activity
6000
5000
4000
3000
2000
1000
0
AP-1
Ets1
Efl1
NFATc
NFATp
GATA-3
—
PMA/cAMP


(c)
-88mIL-5Luc
Relative luciferase activity
1600
1400
1200
1000
800
600
400
200
0
AP-1
AP-1+Ets1
AP-1+Elf1
AP-1+NFATc
AP-1NFATp
AP-1+GATA-3

(JCSMR) and recombinant Ets1 was provided by I. Kola (Monash University). The EMSA showed that the recombinant AP-1 bound strongly to an oligonucleotide encompassing the AP-1 and Ets/NFAT sites of the promoter (Fig.3.12, lane 2). This binding was sequence-specific as it was competed away by an excess of the same unlabelled oligonucleotide (lane 10) but not by an unrelated oligonucleotide (lane 3). Also, mutation of the AP-1 site abolished binding of recombinant AP-1 (lane 6) but mutation of the Ets/NFAT site had no effect (lane 8). By contrast, the binding of recombinant Ets1 to the oligonucleotide encompassing the AP-1 and Ets/NFAT sites was much weaker (lane 4) although still detectable. This binding was also shown to be sequence-specific using the same criteria used for AP-1 binding above. The recombinant Ets1 was shown to bind strongly with a consensus oligonucleotide for Ets (lane 12) but not when the Ets/NFAT site was mutated (lane 13). Therefore the weak binding reflects a property of the Ets binding site of the IL-5 proximal promoter under the conditions used. Weak binding of Ets1 to the analogous Ets/NFAT binding site of the proximal GM-CSF promoter has been observed previously (Thomas et al., 1997). In the present work, AP-1 and Ets1 were also added together but no evidence of the formation of an AP-1/Ets1 complex was obtained (data not shown).

3.2.9. Effect of HMGI(Y) on IL-5 gene expression

The HMGI(Y) proteins, architectural transcription factors, can bind to the minor groove of A/T-rich DNA, and interact directly with several families of transcription factors including NF-kB, AP-1 and NFAT (Thanos et al., 1992; Bustin et al., 1996; Himes et al., 1996, 2000) There are several reports regarding the involvement of HMGI(Y) proteins in the regulation of cytokine gene expression,

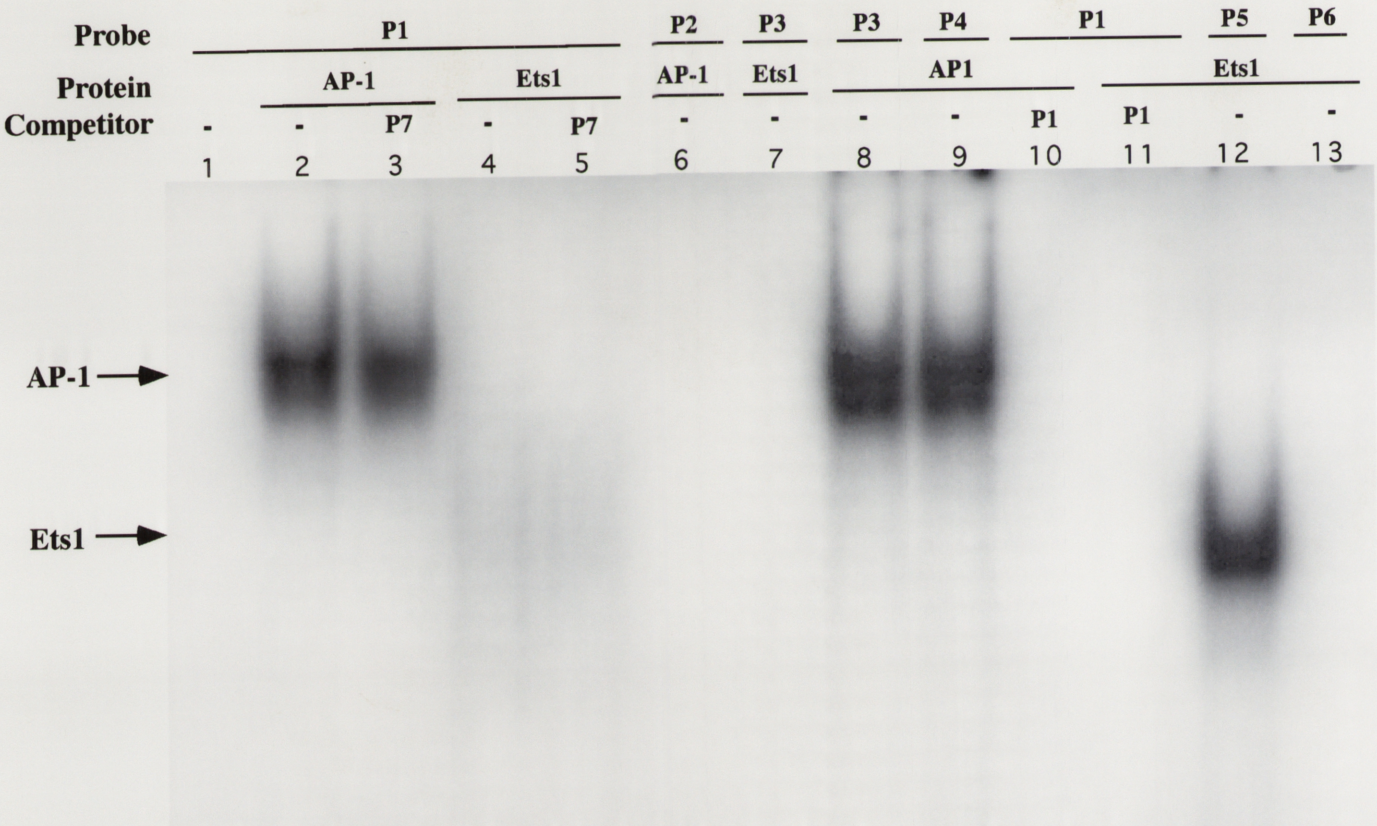


Fig.3.12 Binding of AP-1 and Ets1 ptoteins to the elements of proximal region of mouse IL-5 promoter. The radiolabelled oligonucleotide probe containing the AP-1 and Ets binding sites of the mouse IL-5 promoter (bp -60 to -32) was incubated with 15ng of recombinant AP-1(c-Jun and c-Fos) or Ets1 protein. Unlabeled competitor oligonucleotides (100-fold molar excess over the probe) corresponding to the wild-type IL-5 probe or mutants containing nucleotide substitutions in the AP-1 (P2) or Ets (P3) site (see Materials and Methods) were added to the binding reaction mixtures as indicated. Binding reactions were fractionated on a native polyacrylamide gel and the dried gel autoradiographed at room temperature overnight. Specific protein-DNA binding is indicated with the arrows. P1: wt IL-5 promoter; P2: IL-5 AP-1 mutant; P3: IL-5 Ets mutant; P4: AP-1 consensus; P5: Ets consensus; P6: Ets consensus mutant; P7: non-specific sequence.

including expression of IFN-β, IL-4, IL-2 and GM-CSF (Chuvpilo et al., 1993; Himes et al., 1996; Klei-Hessling et al., 1996; Yie et al., 1997). The IL-5 proximal promoter region is rich in A/T base pairs and has some potential binding sites for HMGI(Y) with A/T sequences present within the GATA site and adjacent to the AP-1 and Ets/NFAT sites. To study if HMGI(Y) can play a role in the regulation of the IL-5 gene, transient transfection experiments were carried out. The mouse IL-5 reporter gene construct was cotransfected with a plasmid expressing HMG I or a plasmid expressing antisense HMG I into D10W cells. The expression of antisense HMG I RNA slightly enhanced, in a dose dependent manner, IL-5 promoter activity in PMA/cAMP-treated D10W cells, (Fig.3.13). The antisense HMGI gave stronger effects on expression of the -88mIL-5Luc construct than on the -1170mIL-5Luc. Surprisingly, overexpression of HMG I protein also resulted in an increase in IL-5 promoter activity in response to PMA/cAMP (Fig.3.13). To identify potential binding sites for HMG I in the IL-5 promoter region, EMSA and DNase footprinting experiments were performed in collaboration with M Bunce using recombinant HMG I protein. Two potential HMGI(Y) binding sites were found in the regions -76 to -88 and -44 to -65 (M. Bunce, unpublished data). The functional role of the site at -76 to -88 was then tested by mutation (Fig.3.14). A transfection experiment showed that the mutation of this potential HMGI(Y) binding site resulted in an increase of IL-5 promoter activity in response to PMA/cAMP stimulation (Fig.3.14). This data supported the results of the antisense experiment. Clearly, further work is required to investigate the function of HMGI(Y) in this system but these results raise the interesting possibility that HMGI(Y) may function as a negative regulator of IL-5 gene expression and as an architectural transcription factor for the proximal promoter region.

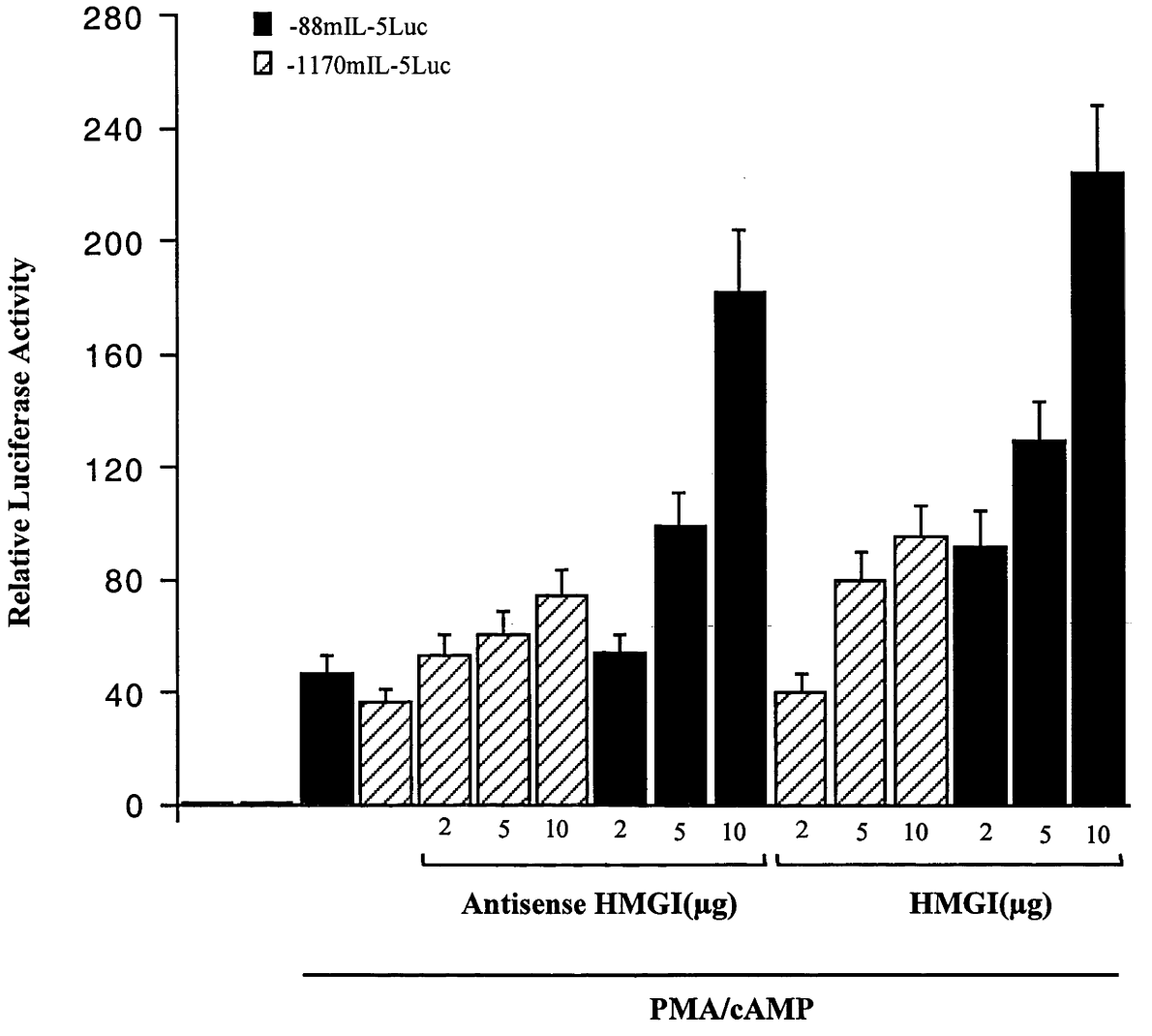


Fig. 3.13 Effect of HMGI on mouse IL-5 expression. The -1170mIL-5Luc or -88mIL-5Luc constructs were cotransfected with varying amount of plasmids expressing HMGI protein or antisense HMGI RNA into D10W cells. Transfected cells were stimulated with PMA and cAMP and luciferase activity was measured (see Materials and Methods). The results represent the average of at least three independent experiments.

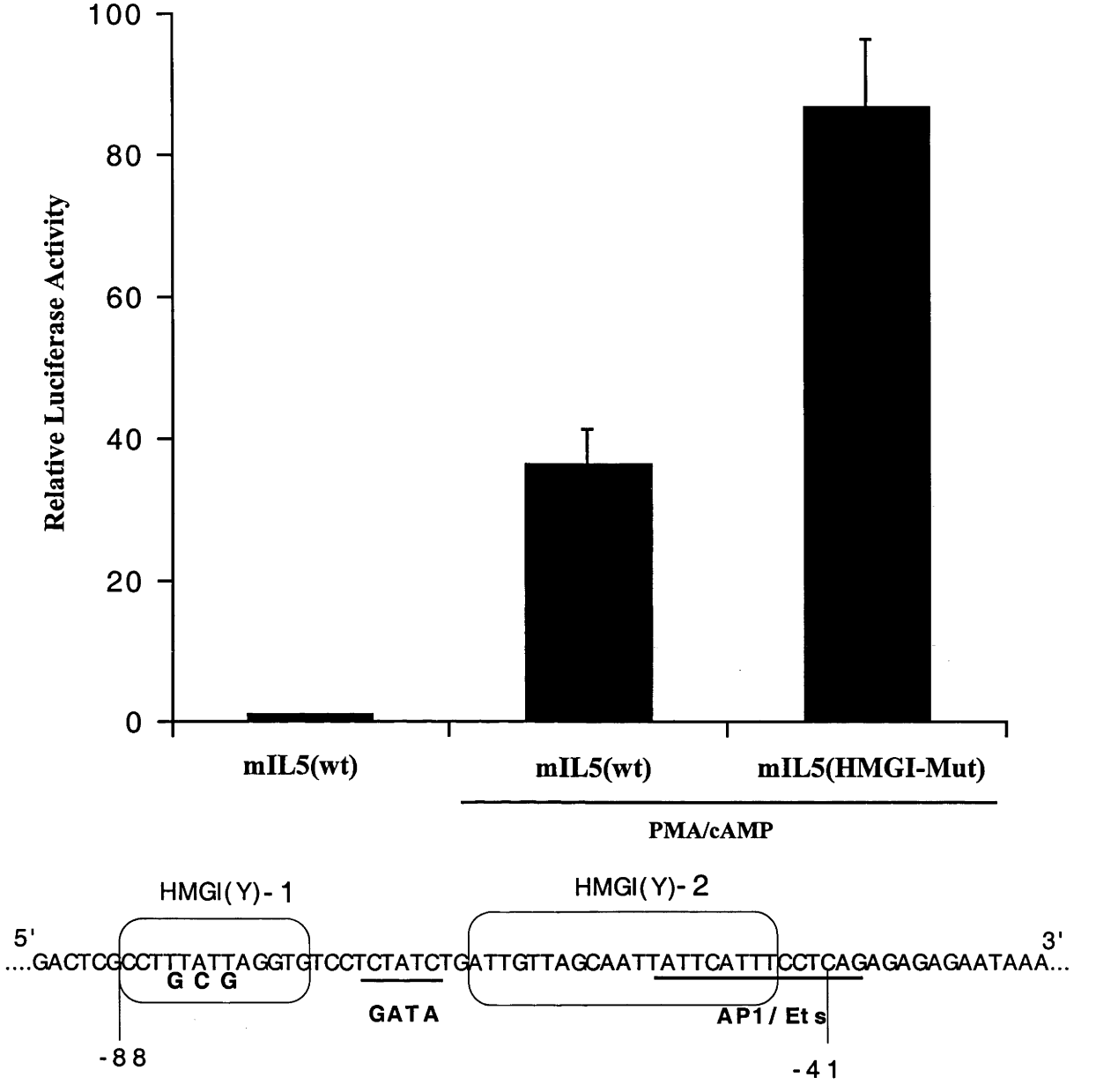


Fig. 3.14 Effects of mutation of HMGI(Y) -1 binding site on IL-5 expression. Wild-type and mutant constructs of -1170mIL-5Luc were transfected into D10W cells. Transfected cells were stimulated with PMA and cAMP. Luciferase activity was measured (see Materials and Methods). The results represent the average of at least three independent experiments.

3.3. Discussion

3.3.1. Establishment of a transient expression system for IL-5 in D10W cells

In the work described in this Chapter, an IL-5 luciferase reporter construct carrying 1170 bases of upstream sequence was prepared and used in transient transfection experiments with D10W cells. The D10W cell line is a subline of the Th2 clone D10.G4.1 derived in this laboratory which grows without spleen cell stimulation. Careful optimization of the electroporation conditions using a GFP control plasmid enabled 36-fold induction of IL-5 reporter gene expression to be achieved using stimulation by PMA and cAMP. Previous attempts to establish such a transient expression system in this laboratory using the parent cell line D10.G4.1 and an analogous reporter construct employing the CAT reporter gene were not successful (Bourke et al., 1995). This was probably because D10.G4.1 is poorly transfectable compared to D10W, the CAT reporter gene has a lower sensitivity of detection than luciferase and PMA was used for stimulation rather than the much more effective combination of PMA/cAMP.

In transient transfection experiments, the -1170mIL-5Luc reporter gene construct was found to be slightly inducible by PMA, more so by cAMP, but strongly synergistically stimulated by PMA/cAMP (Fig.3.3). These results are closely similar to those obtained with stable transfection (Tan, 1998; Young et al., 1999). In contrast to stable transfection, the induction of -1170mIL-5Luc construct in transient transfection was found not to be repressed by dexamethasone (data not shown), suggesting a requirement for a normal chromatin structure for dexamethasone

repression of IL-5 expression. When a reporter construct deleted to -88 bp was transiently transfected into D10W cells, strong induction of expression of the IL-5 gene was also achieved (Fig.3.10) indicating that this region contains the critical regulatory elements involved in the induction of expression of the IL-5 gene. Interestingly, the reporter gene construct carrying -88 bp of IL-5 gene, unlike that carrying -1170 bp of IL-5 gene, is not repressed by dexamethasone when stably transfected into D10W cells (Tan, 1998; Young et al., 1999). Thus, although the proximal promoter region is sufficient for efficient induction of the IL-5 gene by PMA/cAMP, elements further upstream affect its repressibility by dexamethasone.

3.3.2. Transactivation of the IL-5 promoter

Transactivation by AP-1 (c-Fos/c-Jun) gave 400-fold stimulation of IL-5 expression in the absence of stimulation (Fig.3.5a) suggesting that in the Th2 clone D10W, low endogenous levels of AP-1 limit IL-5 gene expression. This is the first demonstration that upregulaton of AP-1 is sufficient to induce the IL-5 promoter without stimulation. Further evidence for the normal involvement of AP-1 in IL-5 gene regulation was provided by the strong inhibitory effect of a dominant negative AP-1 expression construct (Fig.3.7b and c). Elevation of AP-1 levels when Th2 lymphocytes are stimulated may therefore be an important part of the process of IL-5 gene induction and may explain why IL-5 transcription requires protein synthesis. There are two other reported cases where upregulation of AP-1 also appears to be involved in gene induction. The cAMP stimulation of dopamine β-hydroxylase gene expression is mediated by AP-1 (c-Fos, c-Jun and JunD) with c-Fos transcription being up-regulated by cAMP (Swanson et al., 1998). Also, insulin activation of the

AP-1 transcriptional complex is mediated by the ERK MAP kinase pathway and involves stimulation of expression of c-Fos, Fra1 and c-Jun (Griffiths et al., 1998)

A number of studies have detected elevation of AP-1 binding to the CLE0 site of the mouse IL-5 promoter following gene induction (Naora et al., 1994a; Siegel et al., 1995; Karlen et al., 1996; Zhang et al., 1997; Tan, 1998; Young et al., 1999). The present results indicate the involvement of a Jun/Fos heterodimer at the AP-1 site in the IL-5 promoter and show that c-Jun and c-Fos have high functional activity in transactivation. Other laboratories have attempted to define the AP-1 family members binding to the AP-1 site using supershift EMSA experiments. It has been reported that the members of the AP-1 family binding to the IL-5 promoter included JunB, JunD, c-Jun and one or more members of the Fos family including c-Fos, FosB, Fra1 or Fra2 using EL-4 nuclear extracts (Siegel et al., 1995) and Karlen et al., (1996) showed binding of c-Fos and JunB using nuclear extracts of EL-4. Zhang et al., (1997) reported binding of JunB and JunD using nuclear extracts of D10.G4.1.

Transactivation by Ets1 and GATA-3, the other two transcription factors likely to be involved in IL-5 regulation, was low by comparison with AP-1 but significant levels of transactivation were achieved after stimulation with PMA/cAMP. Although Ets1 was more active than Elf1 and NFATp in the transactivation experiments it was of comparable activity to NFATc. However, AP-1 synergized strongly with Ets1 in the absence of stimulation. This synergy was not seen with Elf1, NFATc or NFATp which could also potentially bind to the Ets/NFAT site, suggesting an involvement of Ets1 in normal IL-5 gene induction. Ets1 has been shown to be involved in GM-CSF regulation, where it can cooperate with AP-1 and NF-kB to synergistically transactivate the GM-CSF promoter in Jurkat cells (Thomas et al., 1997). Ets proteins

can physically associate with AP-1 in activated T cells (Bassuk and Leiden, 1995). This Ets and AP-1 interaction is mediated by the binding of the basic domain of Jun to the Ets domain of Ets proteins. Jun, in association with Ets, is capable of interacting with Fos family members to form a trimolecular protein complex. The physical association between Ets1 and AP-1 protein is required for the transcriptional activity of enhancer elements containing adjacent Ets and AP-1 binding sites (Bassuk and Leiden, 1995). Several studies showed that functional interactions between AP-1 and multiple Ets proteins and EMSA studies have demonstrated that complexes of Ets and AP-1 proteins bound to naturally occurring enhancer motifs (Wang et al., 1994; Gottschalk et al., 1993; Wasylyk et al., 1990; Bergelson and Daniel, 1994). Bassuk et al., (1995) demonstrated direct physical interaction between Ets and Jun proteins which helps to explain the molecular basis of the previously observed functional cooperativity between these two important families of transcription factors. Blumenthal et al., (1999) reported that both Ets1 and Ets2 participate human IL-5 gene regulation in Jurkat cells.

EMSA results showed that recombinant AP-1(c-Jun/c-Fos) strongly binds to the AP-1 site within the CLE0 element of the IL-5 promoter. This binding was sequence-specific. The transactivation and EMSA results demonstrated the critical role of the AP-1 site and AP-1(c-Fos/c-Jun) transcription factor in the activation of the mouse IL-5 promoter. Specific binding of Ets1 protein to Ets/NFAT site in the proximal region of IL-5 promoter was also demonstrated although this binding was very weak compared to that with a consensus oligonucleotide for Ets. Although the transactivation data showed strong synergy between AP-1 and Ets1, no evidence for an AP-1/Ets1 complex was obtained in the *in vitro* binding experiments.

GATA-4 was only slightly less active than GATA-3 in the transactivation experiments suggesting that it has the potential to function in IL-5 regulation. The GATA family of transcription factors (GATA-1 to GATA-6) bind to the WGATAR (W=A/T; R=A/G) sequence through a highly conserved C4 zinc finger domain (Laverriere et al, 1994). Based on their expression profile, the GATA proteins may be classified functionally as hemopoietic (GATA-1 to GATA-3) or non-hemopoietic (GATA-4 to GATA-6). GATA-3 is expressed primarily in T lymphocytes and in the embryonic brain and has shown to be important in the development of Th2 cells and the expression of Th2 cytokines (Zheng and Flavell 1997; Zhang et al., 1999). GATA-3 rather than GATA-4 is expressed by mouse Th2 lymphocytes and so would normally be involved in IL-5 expression in mouse T cells. In contrast to these findings, selectivity for GATA-4 has been reported for constitutive human IL-5 expression by the ATL-16T cell line (Yamagata et al., 1997).

The individual GATA elements in the double GATA site in the mouse IL-5 promoter are present in an inverse orientation with respect to each other and are located between -70 and -65 and between -65 and -60. The first sequence conforms with WGATAR consensus sequence and the present work shows it is essential for IL-5 expression in D10W cells. The second site has an intact GAT core but has a T instead of A in the +1 position. Overlapping GATA sites have also been previously identified in many erythroid-expressed genes such as the chicken α-globin promoter (Evans and Felsenfeld, 1991). Overlapping and /or multiple GATA sites appear to confer increased GATA binding activity and may play a key role in differential responsiveness to GATA-3 for different genes (Evans and Felsenfeld, 1991; Tsai et al., 1991). There are a number of reports supporting a role for the proximal GATA

elements in mouse IL-5 promoter activity in Th2 cells (Zhang et al., 1997, 1998; Lee et al., 1995, 1998; Siegel et al., 1995; Stranick et al., 1998). Present studies provided further functional evidence, suggesting that the inverted GATA site is critical for IL-5 activation and that GATA-3 is involved. Compared to AP-1, transactivation of the IL-5 promoter by GATA-3 was low in unstimulated D10W cells, suggesting that sufficient endogenous levels of GATA-3 are present in D10W cells to fully support IL-5 gene expression.

3.3.3. Role of the HMGI(Y) proteins in IL-5 promoter activity

HMGI(Y) proteins are small nonhistone nuclear proteins that bind the narrow minor groove of A:T sequence-rich B form DNA and modify gene transcription by bending the DNA and permitting the interaction of the various transcription factors with the transcriptional machinery (Bustin and Reeves, 1996). HMGI(Y) has been shown to play an important role in the transcription regulation of several cytokine genes including IL-2, GM-CSF, IFNβ and IL-4 (Thanos et al., 1992; Chuvpilo et al., 1993; Himes et al.,1996).

HMGI(Y) appears to have an important role in the activation of IFN-β transcription by facilitating the assembly of the IFN-β enhanceosome at several organizational levels. HMGI(Y) binds *in vitro* to four sites across the IFN-β promoter and promotes the binding of NF-kB and ATF/c-Jun complexes to their binding elements (Thanos and Maniatis, 1992, 1995). Studies on IL-2 regulation indicated a strong involvement of HMGI(Y) in IL-2 activation. HMGI(Y) binds to many sites across the proximal promoter region of IL-2 (Himes et al., 1996). Functional studies using antisense expression for HMGI(Y) RNA showed that HMGI(Y) was a positive

activator of the IL-2 promoter (Himes et al., 1996). Most of the HMGI(Y) binding sites are overlapping or close to known functional transcription factor sites. HMGI(Y) could affect the *in vitro* binding of many of the major transcription factor families that drive IL-2 transcription, including NFAT, NF-kB, and AP-1 (Himes et al., 1996; Shang et al., 1999; Himes et al., 2000).

Studies on the role of HMGI(Y) in IL-4 gene expression and IL-4 signal transduction have shown the importance of phosphorylation in HMGI(Y) activity. There are several HMGI(Y) binding sites in the IL-4 promoter (Chuvpilo et al., 1993). These sites are associated with functional binding sites for NFAT or NFAT/AP-1 complexes and Oct proteins (Chuvpilo et al., 1993). HMGI(Y) inhibits the binding of NFAT factors to specific regions of the IL-4 promoter (Klein-Hessling et al., 1996). Not only can HMGI(Y) inhibit the binding of NFATp but if NFATp is in excess the opposite effect occurs, implying mutually exclusive binding of these two factors.

In the proximal promoter region up to -88 bp of the IL-5 promoter, there are a number of regions which could potentially bind HMGI(Y). Preliminary experiments have been carried out in collaboration with M. Bunce (JCSMR) and it has been shown that recombinant HMGI binds at two sites in the -88 region. In the work described in this Chapter, mutation of the site at (-88 to -77) resulted in an increase in IL-5 transcriptional activity in agreement with the results of antisense experiments (Fig.3.14). These results suggest that like the IL-4 promoter, HMGI(Y) may act as a repressor of IL-5 gene expression. However, these results do not agree with the effect of overexpression of HMGI which also resulted in increased IL-5 transcription (Fig.3.13) indicating a need to more experiments to clarify this issue.

The absolute requirement for the GATA, AP-1 and Ets/NFAT sites for IL-5 gene induction and the synergy demonstrated between AP-1 and Ets in the present work raises the possibility that mouse IL-5 expression may require the assembly of a three dimensional nucleoprotein complex (enhanceosome) analogous to that characterized for the IFN-β gene. Such a complex would be likely to also involve architectural proteins like HMGI(Y) as well as co-activators. Verification of the possibility that the proximal promoter region of the IL-5 gene participates in enhanceosome formation requires further progress in the identification of the coactivators and architectural proteins involved and is an interesting challenge for future research.

Chapter 4 Synergistic Transactivation of the Human Promoter by Transcription Factors AP-1, Ets1 and GATA-3 in Human HSB-2 T cells

4.1. Introduction

The work described in Chapter 3, together with studies from other laboratories, have given a basic understanding of the regulation of the mouse IL-5 gene at least terms of the transcriptional factors and regulatory elements involved. The human IL-5 promoter has in general been less well studied (Chapter 1), largely because of the lack of suitable models. Although it is likely that human IL-5 gene regulation will be similar to the regulation of the mouse IL-5 gene and several studies support this (Chapter 1), the importance of IL-5 in eosinophilic inflammation in asthma, allergic rhinitis, allergic-skin disorders (Rothenberg, 1998) and in gastro-intestinal allergies (Rothenberg et al., 2001) makes IL-5 gene regulation important clinically. Until recently, there was no suitable T cell line which showed inducible IL-5 gene expression. The PER-117 leukemia line (Kees et al., 1994) was recently shown to give inducible IL-5 expression (Mordvinov et al., 1999) but it has not been generally available. HSB-2, another T cell leukemia line, also potentially provides a model for the study of inducible IL-5 expression (Rolfe et al., 1997, Valentine et al., 1998) but a good transient expression system in this cell line has not previously been developed. IL-5 expression in HSB-2 cells is inducible with PMA and ionomycin and mRNA can be detected by PCR-based nuclear run-on asays (Rolfe et al., 1997). IL-5 expression in these cells is also repressed by CsA and FK506 (Rolfe et al., 1997) and thus shows the basic characteristics of human IL-5 regulation in human T cells.

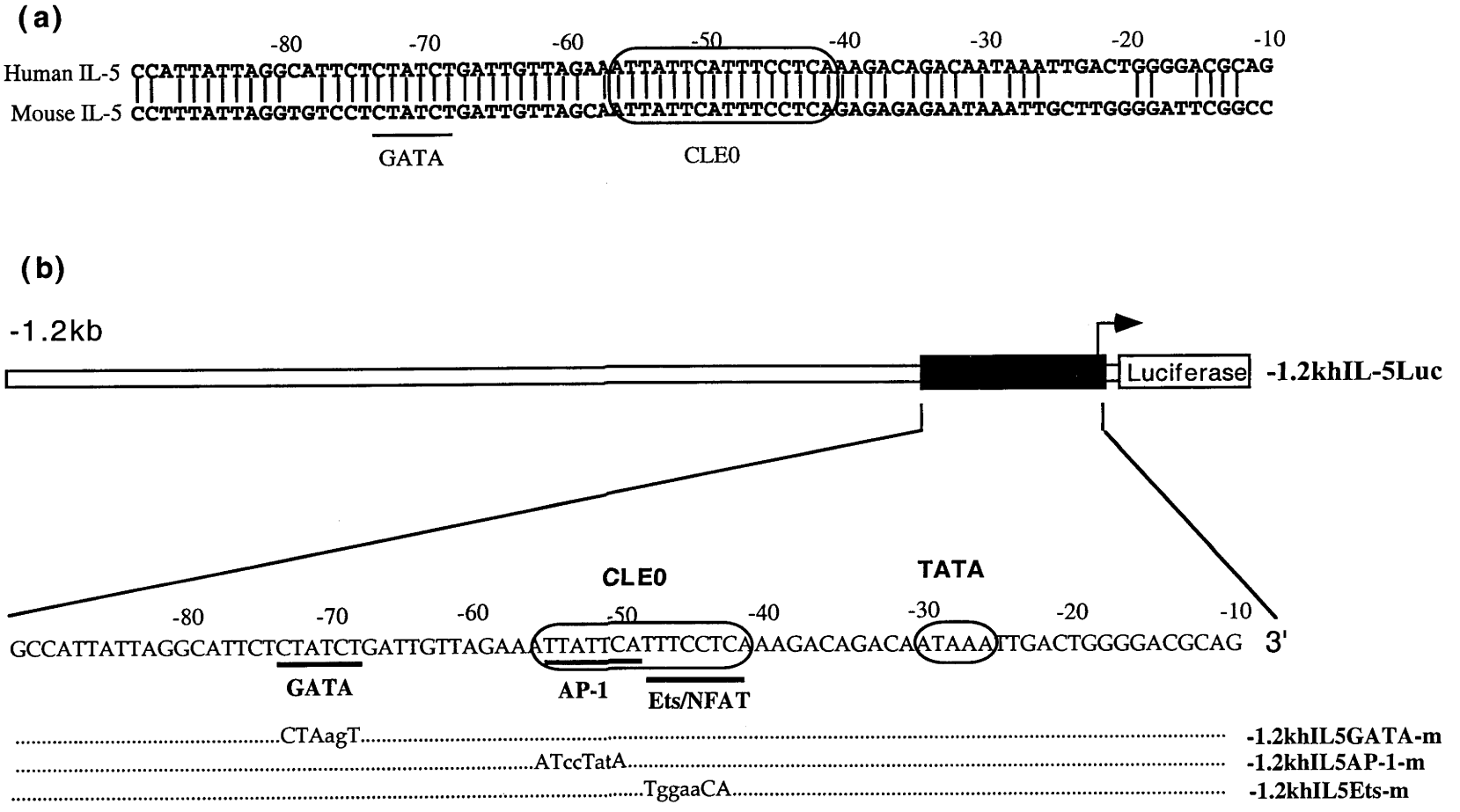


Fig. 4 Structure of the IL-5 promoter. (a) Alignment of the proximal promoter regions of the human and mouse IL-5 genes. (b) Human IL-5 promoter luciferase construct showing the DNA binding sites studied in the present work. The -1.2kb human IL-5 promoter contains a transcriptional control region of approximately 90 bp proximal to the transcriptional initiation site. This region contains the CLE0 element and a GATA transcription factor binding site. -1.2khIL-5Luc is a construct driven by the 1.2kb wild-type human IL-5 promoter.Mutations in the respective binding sites have been introduced into the -1.2khIL-5Luc GATA-m, AP-1-m and Ets-m constructs, as indicated.

In the work described in this chapter, a transient expression system for studying IL-5 gene regulation was established in HSB-2 cells. Transactivation assays were used to study the transcription factors and regulatory elements involved in IL-5 gene induction including synergistic effects. The findings were correlated with the analogous investigations into mouse IL-5 gene regulation described in Chapter 3.

4.2. Results

4.2.1. Expression of human IL-5 luciferase reporter gene in human HSB-2 T cells

In order to develop a sensitive transient reporter gene assay for IL-5 gene expression in HSB-2 cells, both transfection efficiency and cell stimulation were optimized and the sensitive luciferase reporter gene was used. A plasmid encoding GFP under the control of the CMV promoter was used to optimize transfection efficiency in HSB-2 cells. The GFP plasmid was transfected by electroporation into HSB-2 cells using voltages between 220-320 V and a capacitance of 975 μF. Transfected cells were stained with PI (10μg/ml) and analyzed by FACS to determine the number of cells expressing GFP and hence the optimum parameters for electroporation. As shown in Fig.4.1 the optimum conditions were achieved with 280 V at 975 μF where 46 % of the cells were viable and 15 % of the cells expressed GFP.

The optimized electroporation conditions for HSB-2 were utilized to transfect the -1.2khIL5Luc reporter construct into the cells. This construct carries 1.2 kb of upstream sequence of the human IL-5 promoter linked to the luciferase reporter gene.

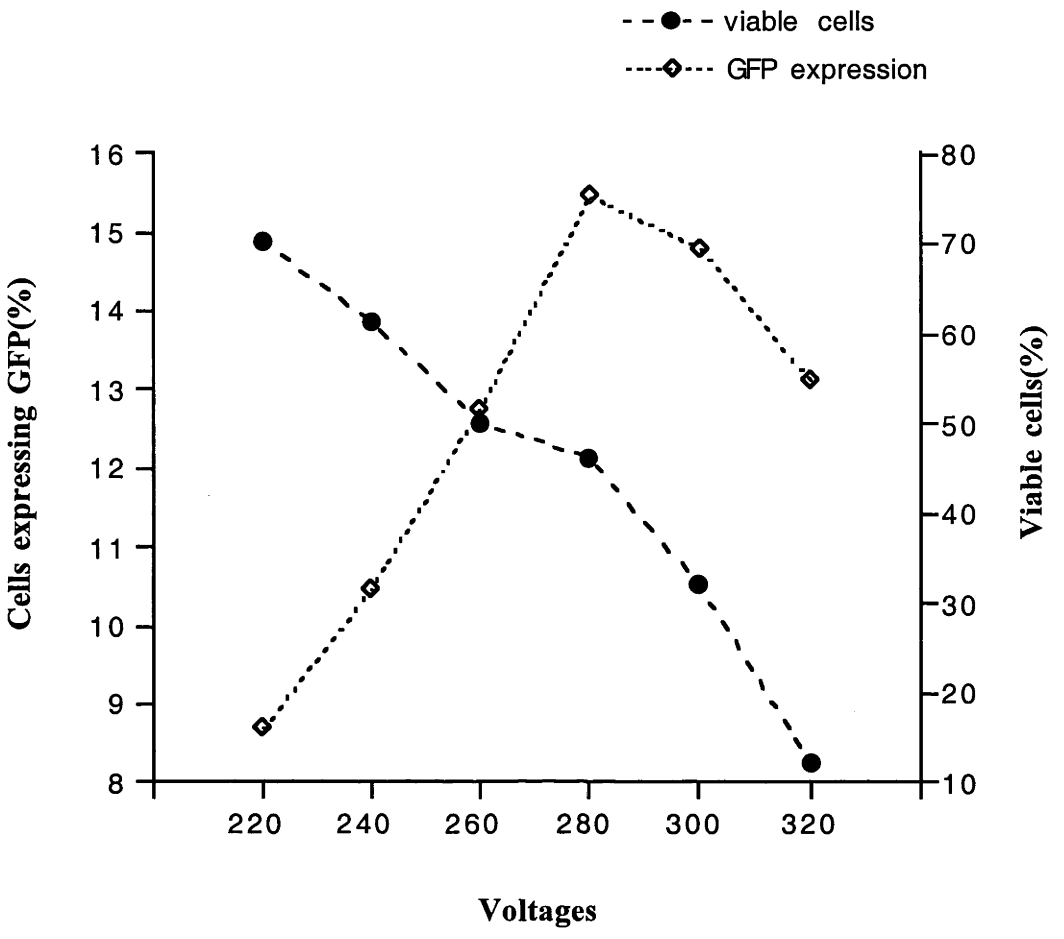


Fig. 4.1 Effect of electroporation voltage on HSB-2 cell viability and transfection efficiency in single pulse electroporation. Five μg of GFP expression construct was transfected into HSB-2 cells using voltages between 220-320 V. After 20 hours recovery , transfected cells were stained with PI. The number of the cells expressing GFP and cell viabilities were determined by FACS analysis (seeMaterials and Methods).

The transfected cells were stimulated with PMA, cAMP and calcium ionophore (A23187) individually and in combination. The -1.2khIL-5Luc construct was induced 1.7 fold by PMA, 1.9 fold by cAMP and 1.2-fold by ionophore. Pairwise combinations of these agents gave up to 3-fold induction whereas PMA, cAMP and ionophore together induced expression of human IL-5 promoter 5.6-fold in HSB-2 cells (Fig.4.2).

To determine the time course of induction of the -1.2khIL-5Luc construct in HSB-2 cells, the luciferase activity of the transfected cells was measured over 24 hours. The peak of luciferase activity occurred between 9 and 12 hours (Fig.4.3).

4.2.2. The AP-1, Ets/NFAT and GATA binding sites are required for transcriptional activity of the human IL-5 promoter in response to PMA/cAMP/ionophore in HSB-2 cells

Sequence analysis of proximal human IL-5 promoter region showed the presence of adjacent putative AP-1 and Ets/NFAT sites (-42 to -56) analogous to the mouse CLE0 binding element and an upstream GATA regulatory element. These three sites are highly conserved between the human and mouse IL-5 promoters (Fig.4a) and present in the proximal promoter region of human IL-5 gene (Fig.4b). To test whether transcriptional activity of the human IL-5 promoter in response to PMA/cAMP/ionophore stimulation in HSB-2 cells may depend on these three transcription factor binding sites as is the case with the mouse IL-5 promoter (Chapter 3), the respective binding sites were mutated, and the effects upon PMA/cAMP/ionophore-mediated IL-5 transcription measured. Mutation of each of the three sites resulted in little change in the basal level of expression but resulted in

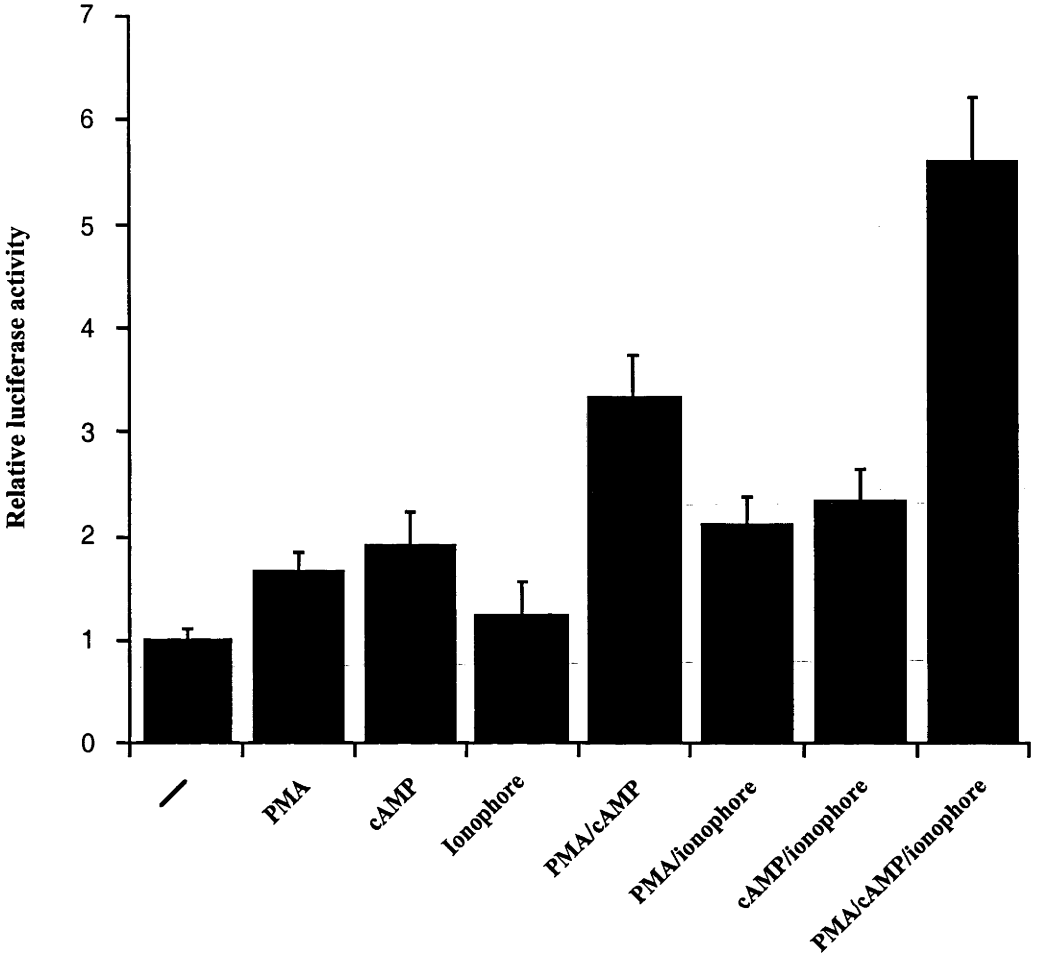


Fig. 4.2 Transcriptional activation of the human IL-5 promoter in HSB-2 cells in response to PMA, cAMP and calcium ionophore. The HSB-2 cells were transfected with the -1.2khIL-5Luc construct. Transfected cells were stimulated with PMA, cAMP and ionophore individually or in combination and luciferase activity was measured (see Materials and Methods). The results represent the average of at least three independent experiments.

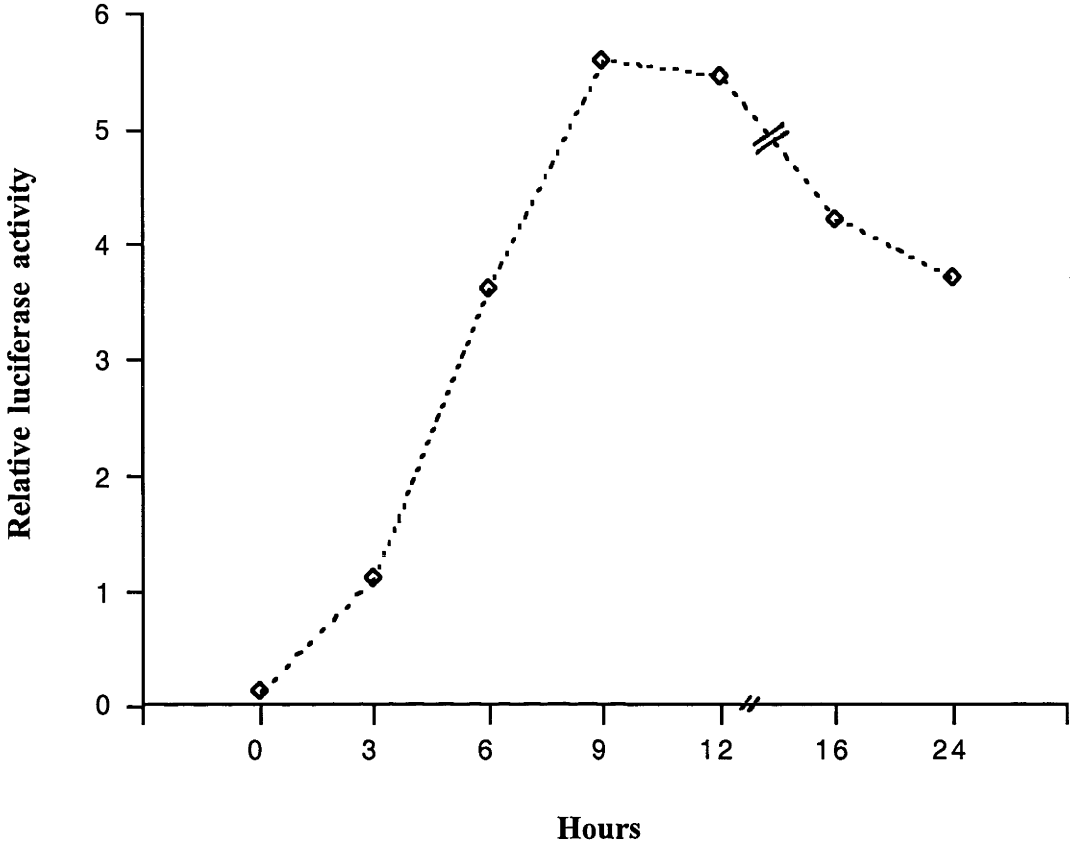


Fig. 4.3 Time course of human IL-5 luciferase reporter gene expression in HSB-2 cells in response to PMA, cAMP and ionophore. HSB-2 cells were transiently transfected with the -1.2khIL-5Luc construct. Transfected cells were treated with PMA/cAMP/ionophore and luciferase activity was measured between 0-24 hours (see Materials and Methods).

almost complete abolition of IL-5 expression in response to PMA/cAMP/ionophore treatment (Fig.4.4), indicating that the AP-1, Ets/NFAT and GATA binding sites are indispensable for induction of the human IL-5 gene in HSB-2 cells

4.2.3. AP-1, Ets1 and GATA-3 transcription factors transactivate the IL-5 promoter in human HSB-2 T cells

To examine which transcription factors are involved in the transcription of IL-5 gene, transactivation experiments were performed using expression plasmids encoding relevant transcription factors. The -1.2khIL-5Luc construct was cotransfected with various expression plasmids into HSB-2 cells to analyse the effect of overexpression of these factors on the transcriptional activity of the human IL-5 promoter.

To test if AP-1, in the absence of stimulation, can transactivate the IL-5 promoter in HSB-2 cells, an expression construct for AP-1 was cotransfected together with the -1.2khIL-5Luc reporter construct into HSB-2 cells. AP-1 expression resulted in an 85-fold increase in transcription of the IL-5 promoter in the absence of stimulation. The transcriptional activity was increased to 500-fold by stimulation with PMA, to 180-fold with cAMP and to 130-fold with ionophore. The combination of PMA, cAMP and ionophore increased the expression of IL-5 promoter to 1000-fold (Fig.4.5a).

Transfection with an Ets1 expression plasmid resulted in a 15-fold increase in transcriptional activity of the IL-5 gene without stimulation (Fig.4.5b). To investigate the requirements for Ets1 transactivation of IL-5, transiently transfected HSB-2 cells were stimulated with PMA, cAMP and ionophore individually or in combination.

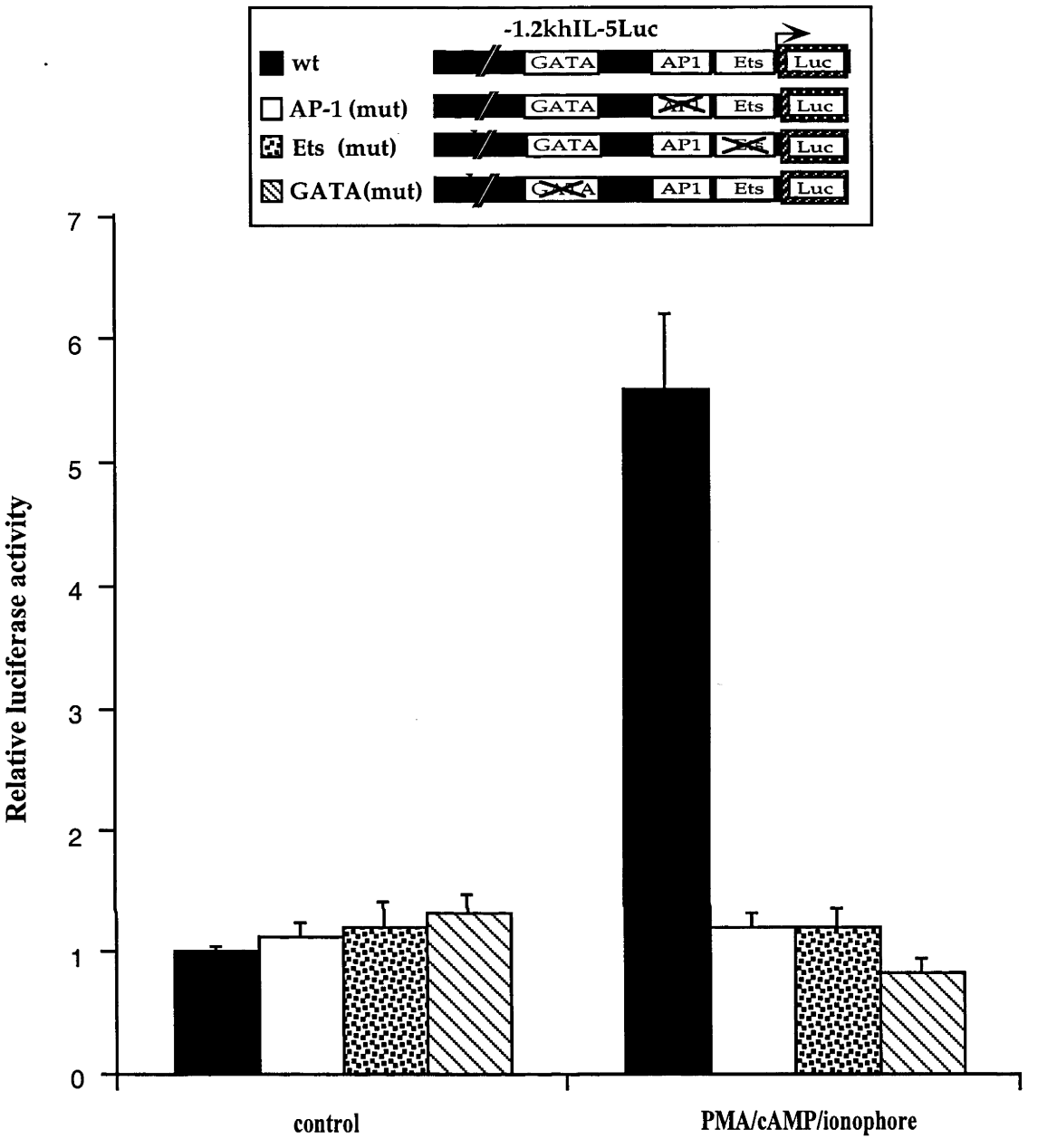


Fig. 4.4 Effects of mutation of the putative AP-1, Ets/NFAT and GATA sites of the human IL-5 promoter on its induction. HSB-2 cells were transfected with the indicated plasmids and then treated with PMA/cAMP/ionophore and luciferase activity was measured (see Materials and Methods). The results represent the average of at least three independent experiments.

PMA treatment alone was able to enhance IL-5 transcription to 35-fold, stimulation with cAMP resulted in 20-fold induction, but ionophore reduced activation of the IL-5 promoter to 8-fold compared to Ets1 transactivation alone. The combination of PMA, cAMP and ionophore gave the highest Ets1 transactivation (136-fold) (Fig.4.5b).

Elf1 is one of Ets family of transcription factors which has been shown to be involved in GM-CSF gene expression (Wang et al., 1995) and this transcription factor could potentially interact at the Ets/NFAT site in the IL-5 promoter. Transactivation experiments with Elf1 only resulted in 2-fold induction of IL-5 promoter activity (Fig.4.5c). Stimulation with PMA, cAMP or ionophore individually had little effect on Elf1-mediated IL-5 transcription. However, when transfected cells were treated with PMA/cAMP/ionophore, the transcriptional activity of the IL-5 promoter was slightly increased (Fig.4.5c). Thus Ets1 is considerably more active than Elf1 in the transactivation of the human IL-5 promoter.

GATA-3 is expressed primarily in T lymphocytes and in the Th2 subclass of T helper cells. Functionally important GATA-3-binding sites have been identified in T cell receptor genes and the CD8 gene (Ho et al., 1991; Henderson et al., 1994). GATA-3 has been reported to be involved in Th2-specific expression of the mouse IL-5 gene (Zhang et al., 1997) and evidence for its involvement was also obtained in the present work (Chapter 3). To investigate the functional role of GATA-3 in human IL-5 expression. HSB-2 cells were cotransfected with the -1.2khIL-5Luc construct and a GATA-3 expression construct. GATA-3 was able to increase IL-5 promoter activity 100-fold in the absence of stimulation (Fig.4.6). When transfected cells were treated with PMA, cAMP or ionophore, 500-fold, 300-fold and 360-fold increases in

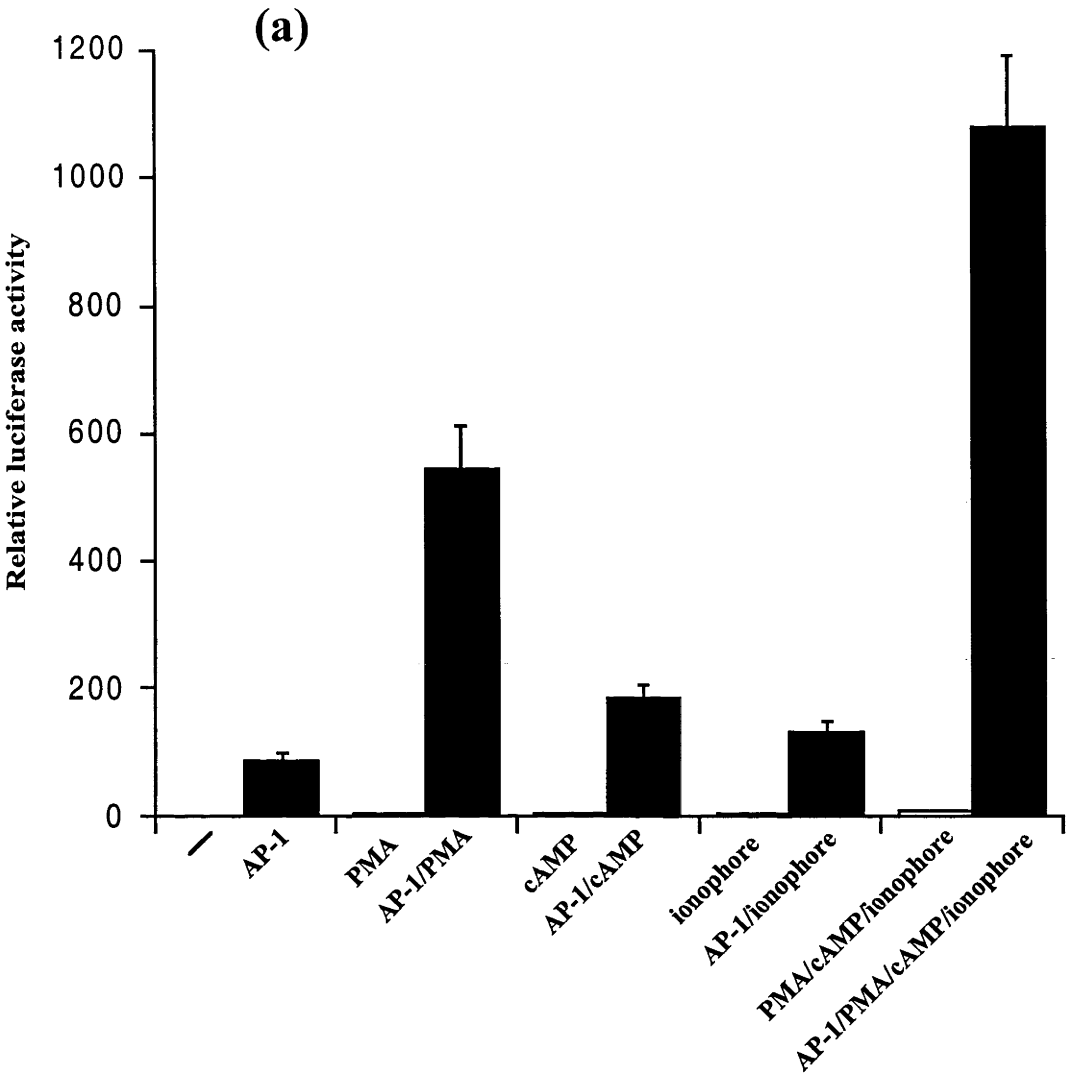
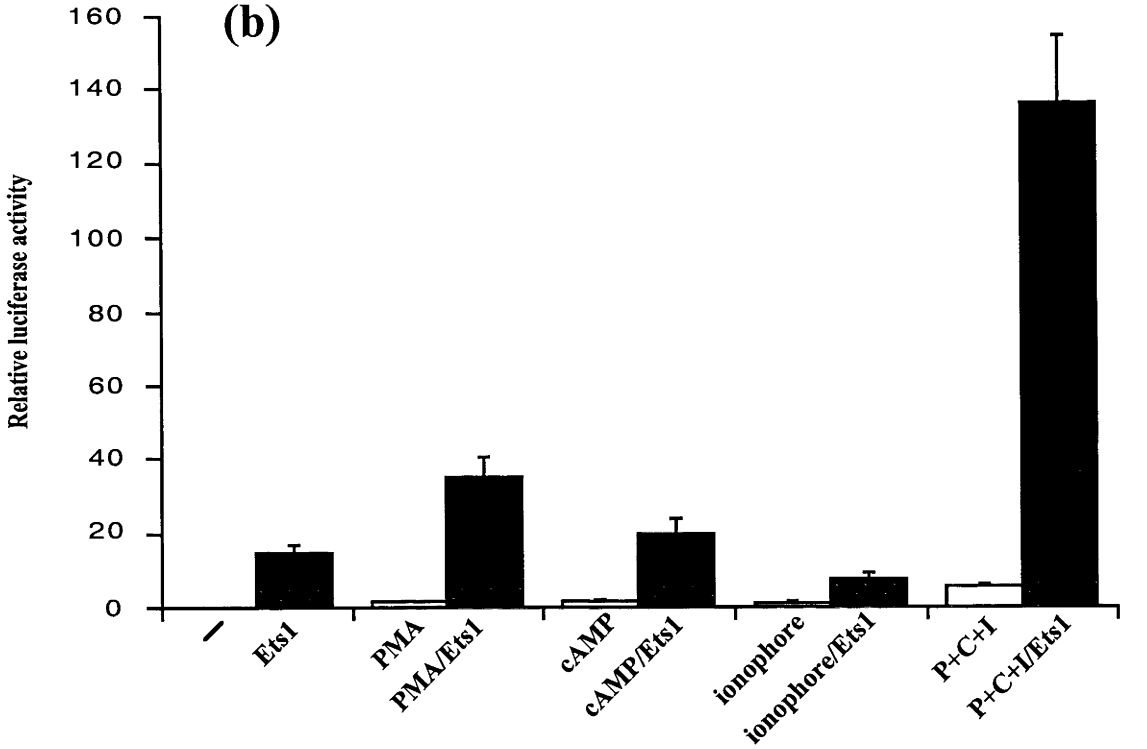
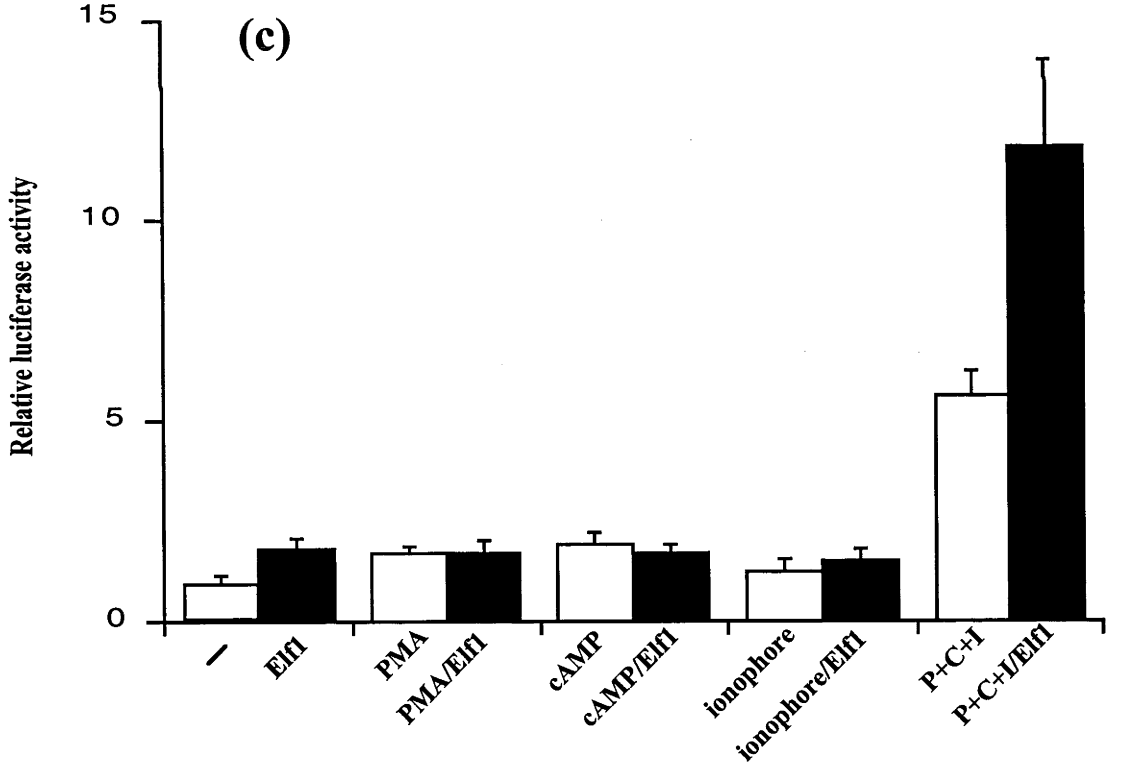
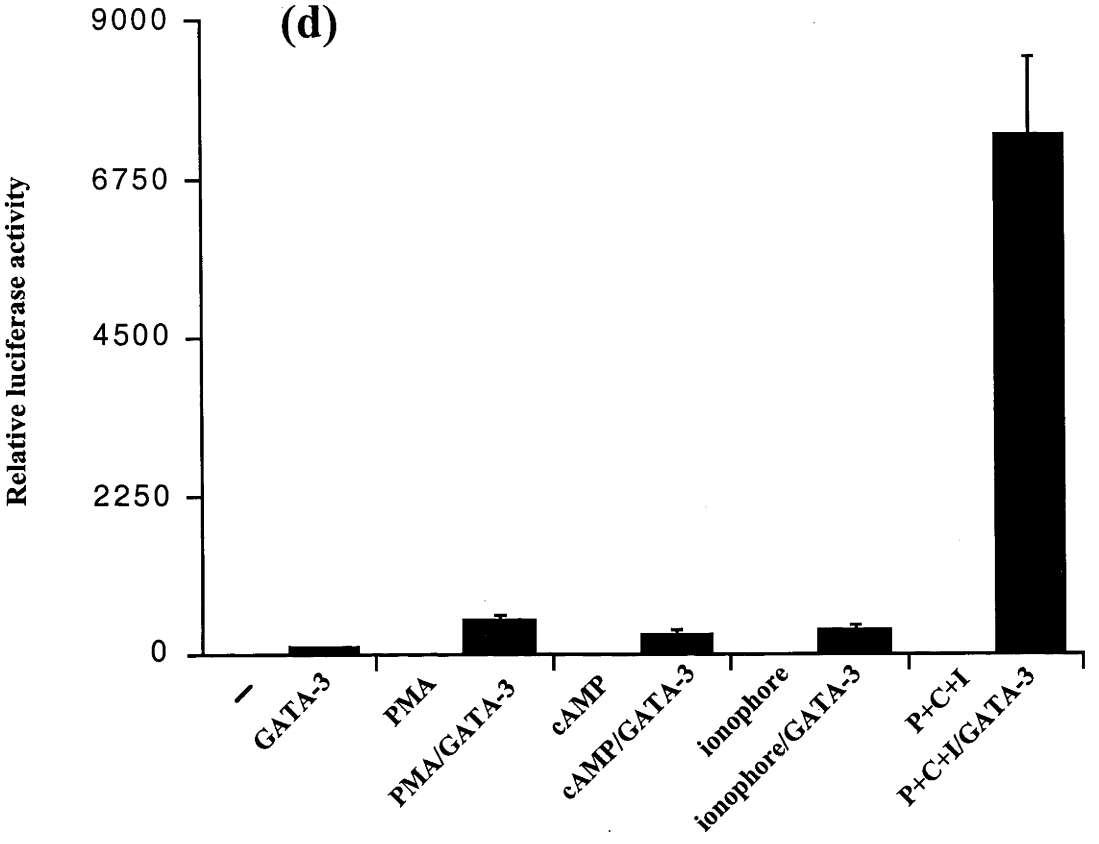
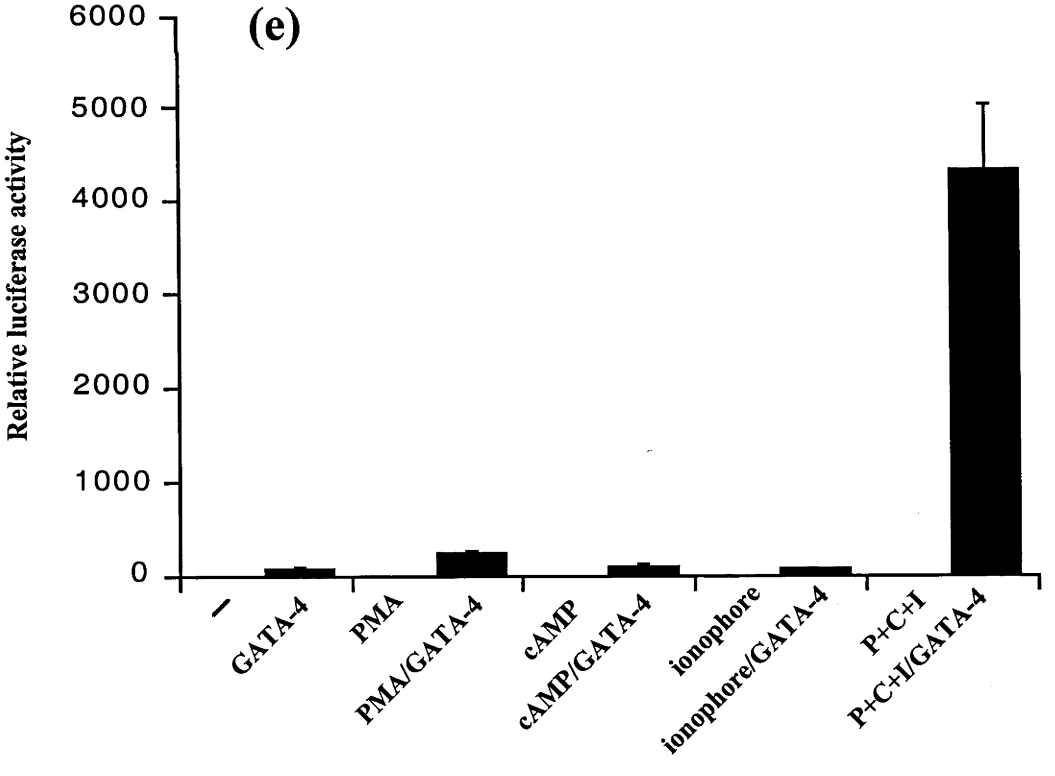


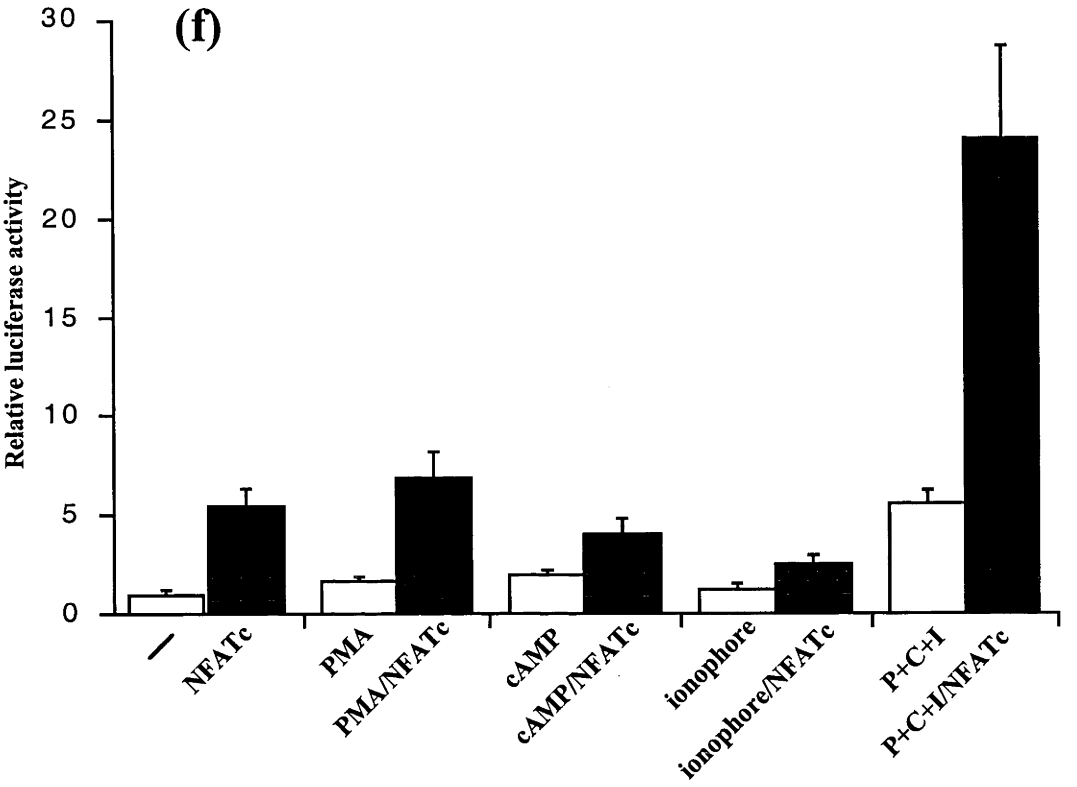
Fig. 4.5 Transactivation of the human IL-5 promoter by relevant transcription factors. HSB-2 cells were cotransfected with the -1.2khIL-5Luc construct and expression vectors encoding different transcription factors. Transfected cells were stimulated as indicated in the figure and luciferase activity was measured (see Materials and Methods). P+C+I denotes PMA + cAMP + ionophore. (a) AP-1; (b) Ets1; (c) Elf1; (d) GATA-3; (e) GATA-4; (f) NFATc; (g) NFATp. The results represent the average of at least three independent experiments.

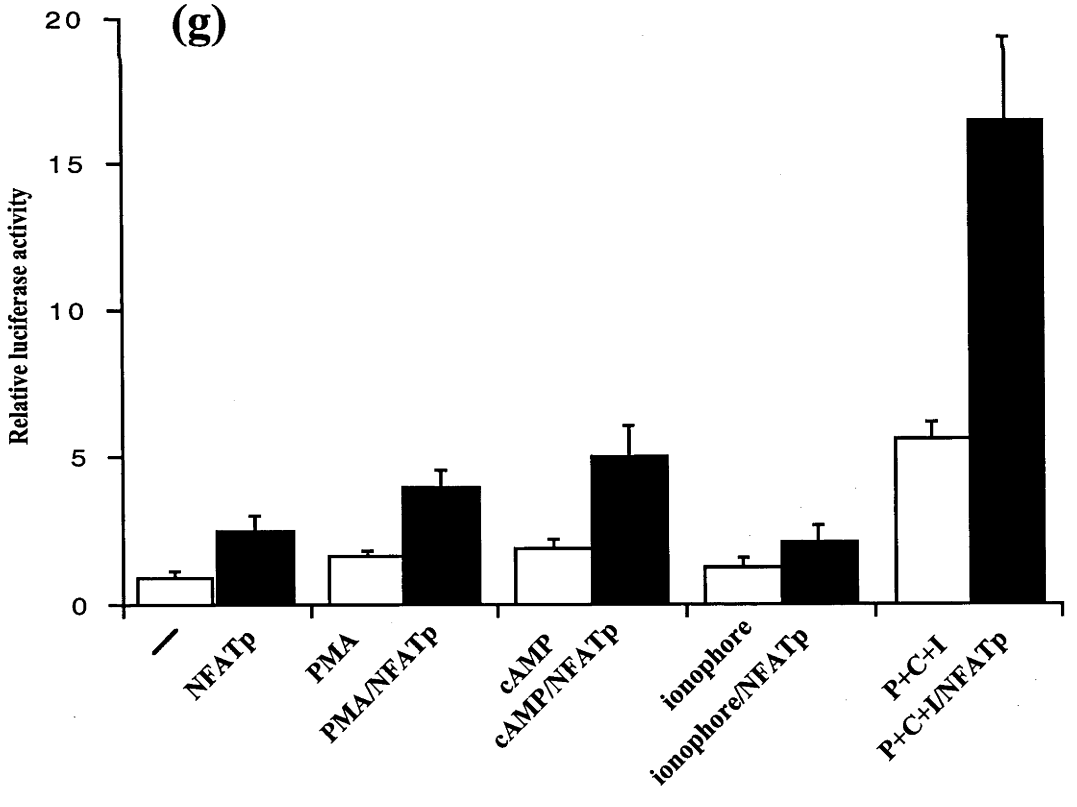

(b)
Relative luciferase activity
160
140
120
100
80
60
40
20
0
–
Ets1
PMA
PMA/Ets1
cAMP
cAMP/Ets1
ionophore
ionophore/Ets1
P+C+I
P+C+I/Ets1


(c)
Relative luciferase activity
15
10
5
0
–
Elf1
PMA
PMA/Elf1
cAMP
cAMP/Elf1
ionophore
ionophore/Elf1
P+C+I
P+C+I/Elf1


(d)
9000
6750
4500
2250
0
Relative luciferase activity
–
GATA-3
PMA
PMA/GATA-3
cAMP
cAMP/GATA-3
ionophore
ionophore/GATA-3
P+C+I
P+C+I/GATA-3


(e)
6000
5000
4000
3000
2000
1000
0
Relative luciferase activity
–
GATA-4
PMA
PMA/GATA-4
cAMP
cAMP/GATA-4
ionophore
ionophore/GATA-4
P+C+I
P+C+I/GATA-4


(f)
Relative luciferase activity
30
25
20
15
10
5
0
—
NFATc
PMA
PMA/NFATc
cAMP
cAMP/NFATc
ionophore
ionophore/NFATc
P+C+I
P+C+I/NFATc


(g)
Relative luciferase activity
20
15
10
5
0
—
NFATp
PMA
PMA/NFATp
cAMP
cAMP/NFATp
ionophore
ionophore/NFATp
P+C+I
P+C+I/NFATp

expression respectively were achieved. Moreover, a 7000-fold increase over basal expression levels was obtained by stimulation with PMA/cAMP/ionophore (Fig.4.5d).

The selective involvement of GATA-4 in transcription of the human IL-5 gene in ATL-16T cells has been reported (Yamagata et al., 1997) although GATA-4 involvement remains controversial since GATA-3 is the GATA family member normally expressed in T cells (Oosterwegel et al., 1992). The effect of GATA-4 on the transactivation of the human IL-5 promoter in HSB-2 cells was therefore tested. GATA-4 was able to transactivate the IL-5 promoter 80-fold in the absence of stimulation (Fig.4.6). PMA-treated cells gave 200-fold induction, while 100-fold stimulation was achieved with cAMP and 65-fold stimulation with ionophore. 4000-fold induction was obtained with a combination of PMA/cAMP/ionophore (Fig.4.5e). These results suggest that even though GATA-4 is not normally expressed in T cells, it can function as effectively as GATA-3 in regulating IL-5 expression.

NFAT has been shown to be important in the regulation of a number of cytokine genes, including GM-CSF, IL-4 and IL-2 (Rooney et al., 1995; Cockerill et al., 1995). Most NFAT binding sites in cytokine promoters are accompanied by neighbouring sites that bind other transcription factors such as members of the AP-1 or its relatives. In the IL-5 promoter, NFAT could potentially be involved in binding to the putative Ets/NFAT site adjacent to AP-1 site in the CLE0 element. It was therefore of interest to study if NFAT can transactivate the IL-5 promoter. Expression plasmids for NFATp or NFATc and the -1.2khIL5Luc reporter construct were cotransfected into HSB-2 cells. NFATc gave 5-fold transactivation of the IL-5 promoter in the absence of stimulation (Fig4.6). Treatment with PMA, cAMP or

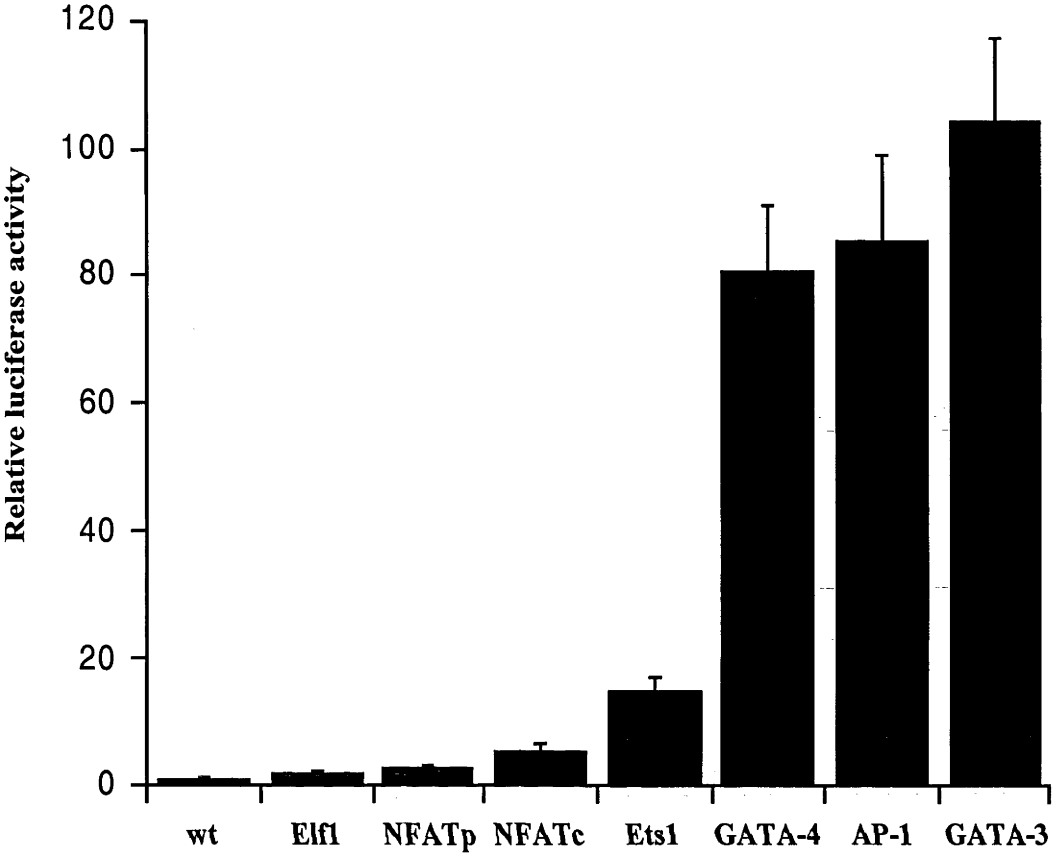


Fig. 4.6 Comparison of the transactivation of the human IL-5 promoter using expression constructs for different transcription factors without stimulation. The -1.2khIL-5Luc construct was cotransfected with various expression constructs encoding relevant transcription factors into HSB-2 cells (see Materials and Methods).The results represent the average of at least three independent experiments.

ionophore did not enhance the activity of the IL-5 promoter but the three agents in combination resulted in a synergistic stimulation of 24-fold (Fig.4.5f). Overexpression of NFATp led to 2.5-fold induction (Fig.4.6) and 15-fold induction in IL-5 promoter activity was observed when transfected cells were treated with PMA/cAMP/ionophore (Fig.4.5g). These results suggest that neither NFATp or NFATc is very active in the transactivation of the human IL-5 promoter compared to Ets1. It seems likely from the above results that Ets1 is the transcription factor functioning at the putative Ets/NFAT site in the human IL-5 promoter.

4.2.4. AP-1, Ets1 and GATA-3 synergistically transactivate the human IL-5 promoter in HSB-2 cells

Since the binding sites for Ets, AP-1 and GATA are relatively close together in the proximal region of IL-5 promoter with the Ets and AP-1 sites being adjacent and since AP-1, Ets1 and GATA-3 individually gave strong transactivation of the IL-5 promoter in HSB-2 cells, it was of interest to examine if these transcription factors can function cooperatively in stimulating IL-5 expression, Expression plasmids for AP-1, Ets1 and GATA-3 were therefore transfected in different combinations together with the -1.2khIL-5Luc into HSB-2 cells. GATA-3 showed synergistic transactivation with Ets1 (400-fold), with AP-1 (1000-fold) and with Ets1/AP-1 (2500-fold) in the absence of stimulation (Fig.4.7). Similar synergistic interactions were shown between GATA-4 and Ets1 and AP-1 (Fig.4.7).

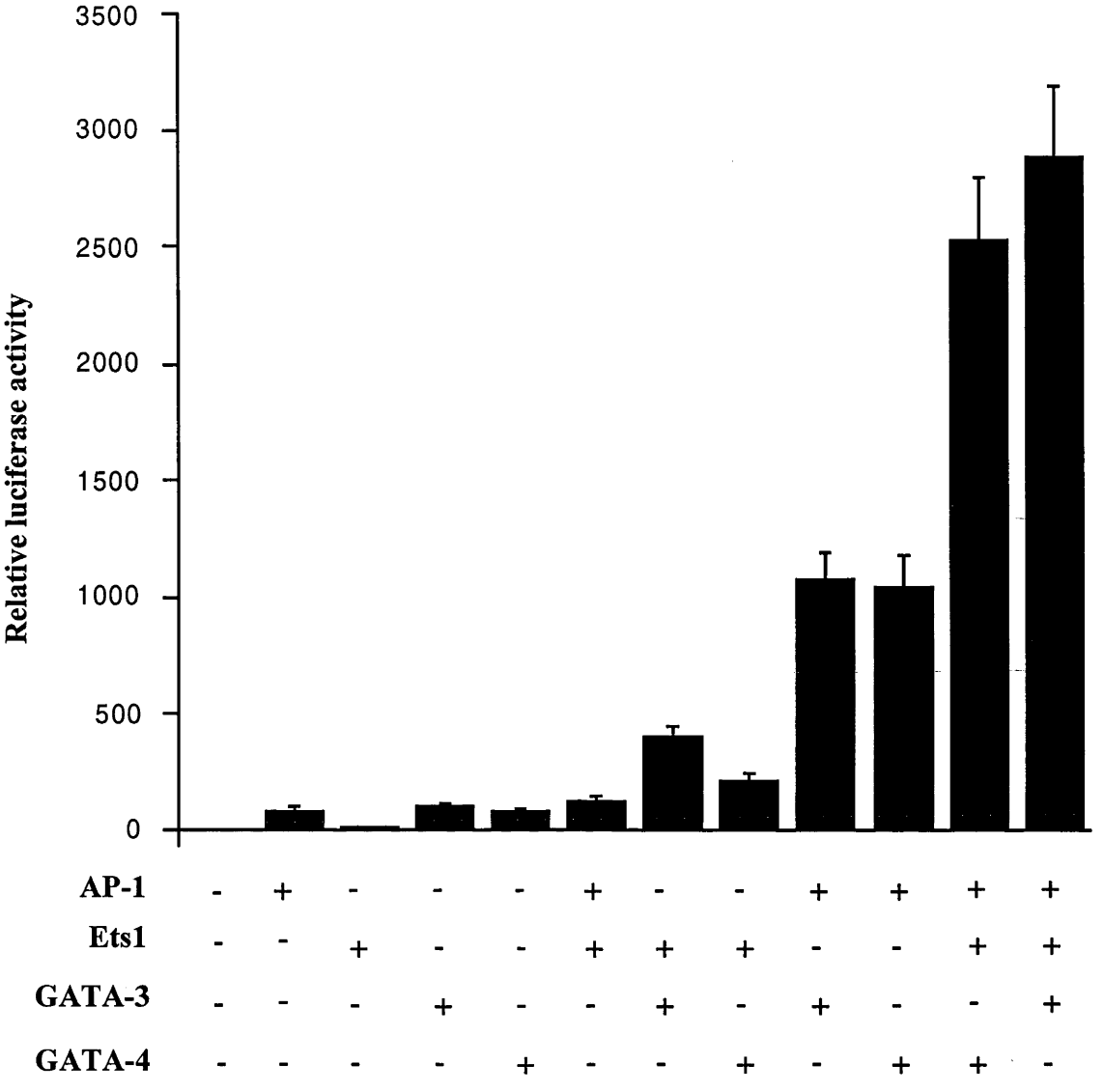


Fig. 4.7 Synergism between transcription factors in the transactivation of the human IL-5 promoter in HSB-2 cells. The -1.2khIL-5Luc construct was cotransfected with a combination of various indicated expression plasmids into HSB-2 cells and luciferase activity was measured (see Materials and Methods). The results represent the average of at least three independent experiments.

4.2.5. Synergistic cooperation between AP-1, Ets1 and GATA-3 is dramatically reduced by mutation of their binding sites.

In view of the high level of transactivation of the human IL-5 promoter achieved in the absence of stimulation, it was considered important to determine if the transactivation was dependent on the three transcription factor binding sites shown to be essential for normal inducible IL-5 gene expression (Fig.4.4). Transfection experiments were therefore carried out with -1.2khIL-5Luc constructs containing mutations of the individual transcription factor binding sites and the effect of AP-1, Ets1 and GATA-3 transactivation determined in human HSB-2 cells. The transactivation activity of GATA-3 on human IL-5 expression was reduced from 100-fold to 9.5-fold by mutation of the GATA site. Similarly Ets1 transactivation was dramatically decreased (14- to 3-fold) by mutation of the Ets site, and mutation of the AP-1 site abrogated the AP-1 transactivation from 85- to 5.6-fold (Fig.4.8). All of these results indicated that the transactivations observed were dependent on binding to the respective transcription factor binding sites in the proximal promoter region. Also, in each case, transactivation by each of the three transcription factors was dependent on the presence of intact binding sites for all three transcription factors (Fig.4.8) as is the case with normal gene induction (Fig.4.4). Similarly the synergistic transactivation of 2800-fold produced by overexpression of AP-1, Ets1 and GATA-3 together in unstimulated cells transfected with -1.2khIL-5Luc was completely abolished by the mutations in each of the transcription factor binding sites (Fig.4.9).

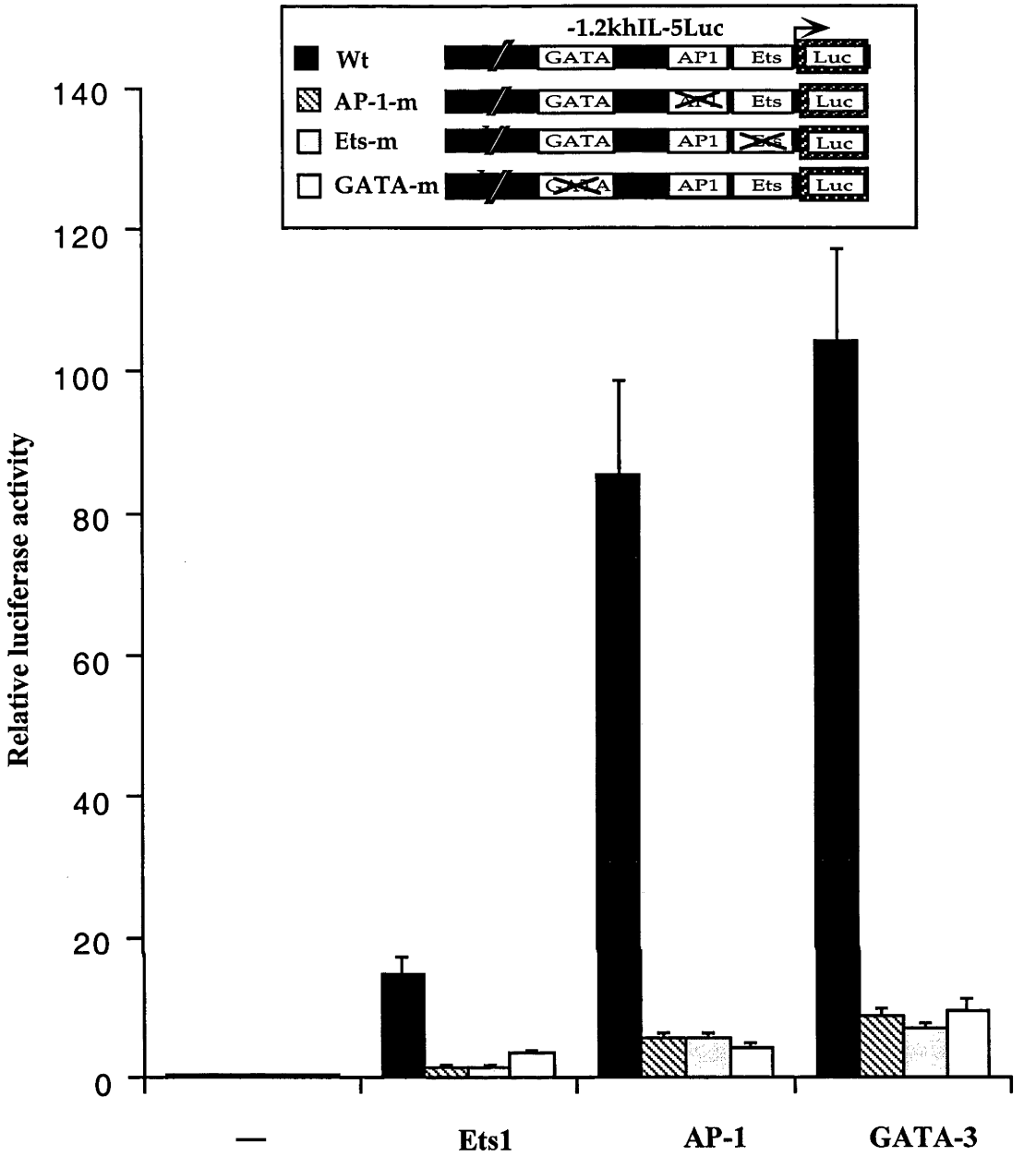


Fig.4.8 Effect of mutation of the transcription factor binding elements in human IL-5 promoter on transactivation by AP-1, Ets1 and GATA-3 in HSB-2 cells. The structures of the mutant human IL-5 promoter constructs are shown schematically. The mutant constructs were cotransfected with expression plasmids for AP-1, Ets1 and GATA-3 into HSB-2 cells as indicated in the figure and luciferase activity was measured (see Materials and Methods). The results represent the average of at least three independent experiments.

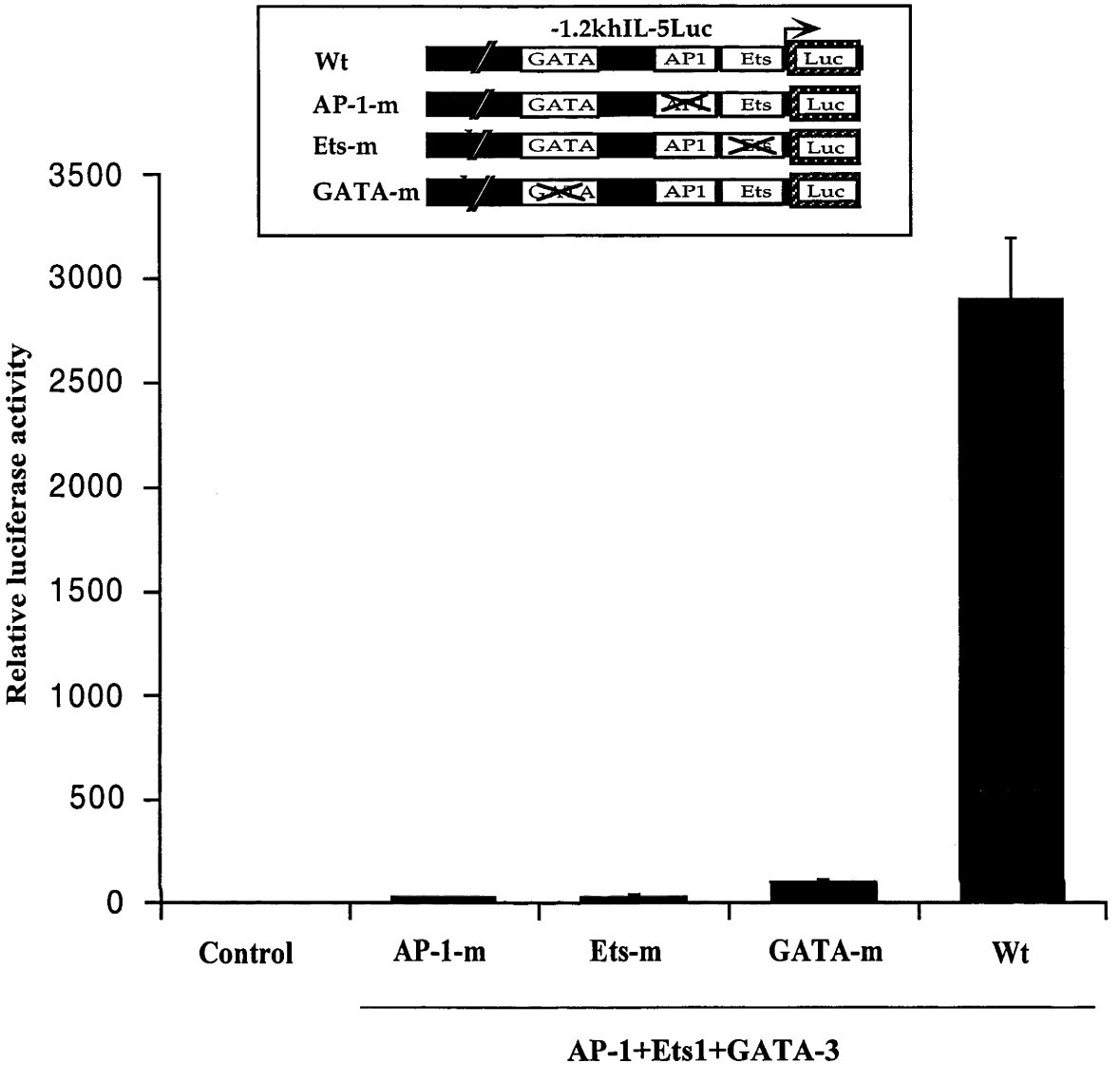


Fig. 4.9 Effect of mutation of the respective transcription factor binding sites on the synergistic transactivation of human IL-5 promoter by AP-1, Ets1 and GATA-3. The HSB-2 cells were cotransfected with wild-type or indicated mutant -1.2khIL-5Luc constructs together with expression constructs for AP-1, Ets1and GATA-3 and luciferase activity was measured (see Materials and Methods). The results represent the average of at least three independent experiments.

4.2.6. Role of AP-1 in human IL-5 promoter activation in HSB-2 cells

Since AP-1 can significantly transactivate the human IL-5 promoter in HSB-2 cells, one might expect that dominant negative AP-1 would reduce the normal induction of the human IL-5 promoter in response to PMA/cAMP/ionophore stimulation. The dominant negative AP-1 used in these experiments was made by fusing an acidic amphipathic extension to the N-terminus of the c-Jun and c-Fos leucine zipper domain (Olive et al., 1997). The acidic extension of c-Jun and c-Fos interacts with the basic region of c-Jun or c-Fos forming a coiled-coil extension of the leucine zipper and thus prevents the basic region of wild-type c-Jun and c-Fos binding to DNA.

Dominant negative AP-1 (DN-AP-1) expression plasmid constructs were cotransfected together with the -1.2khIL-5Luc reporter construct into HSB-2 cells. The transfected cells were treated with PMA/cAMP/ionophore. The repression of IL-5 promoter activity by DN-AP-1 was very significant, and it was also dose-dependent (Fig.4.10) confirming a role for AP-1 in normal human IL-5 gene induction.

4.3. Discussion

The regulation of IL-5 gene induction during T cell activation is a complex process that is controlled primarily at the level of transcription (Naora et al., 1994b). To investigate the molecular mechanisms involved in the regulation of the human IL-5 gene in activated T cells, the proximal region of this promoter was examined in detail. There is a shortage of suitable human T cell lines which show normal inducible expression of the IL-5 gene. For example, human IL-5 gene transcription has been studied in the human Jurkat cell line which does not express IL-5

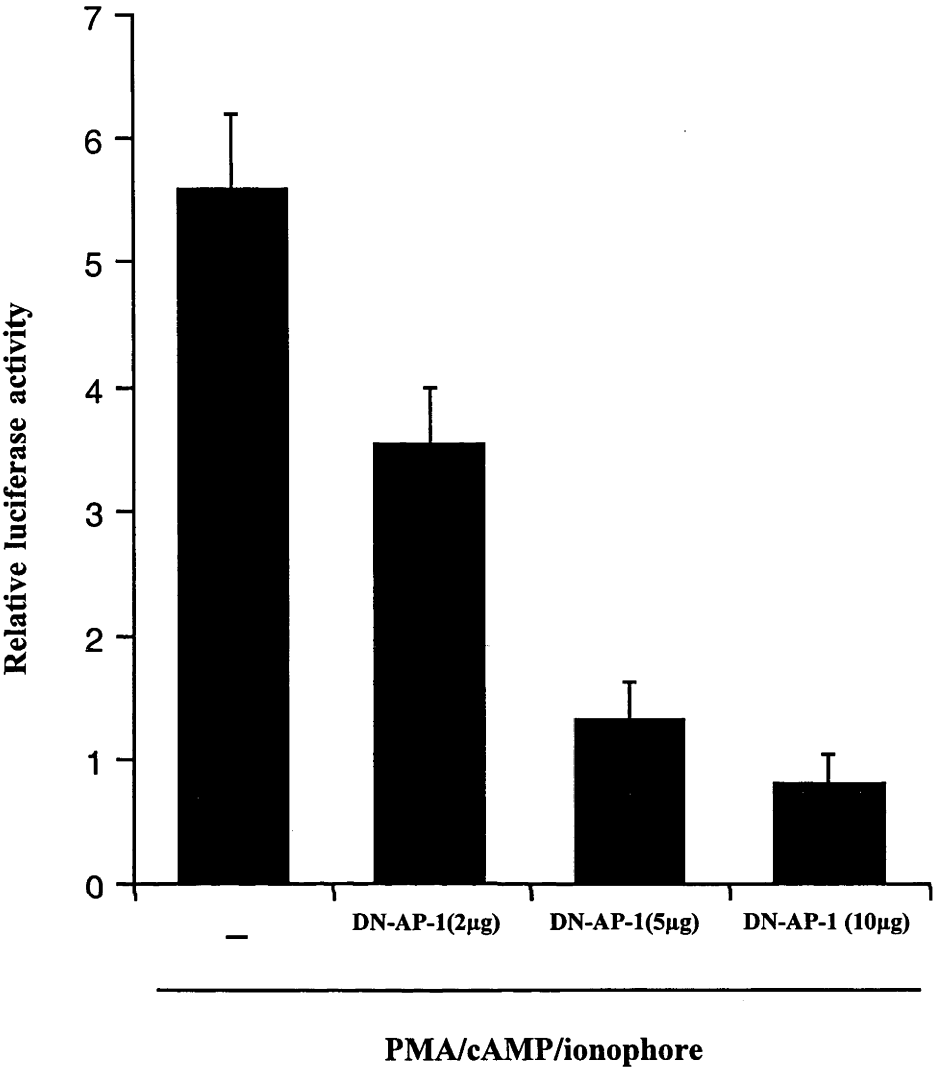


Fig. 4.10 Effect of dominant negative AP-1 on human IL-5 promoter expression. HSB-2 cells were cotransfected with the -1.2khIL-5Luc construct and plasmids expressing dominant negative c-Fos and c-Jun. Transfected cells were treated with PMA/cAMP/ionophore and luciferase activity was measured (see Materials and Methods).The results represent the average of at least three independent experiments.

(Blumenthal et al., 1999), mouse EL-4 cells (Gruart-Gouilleux et al., 1995) and the human T-cell leukemia cell line ATL-16T (Yamagata et al., 1997) which expresses IL-5 constitutively. The human HSB-2 T cell line shows inducible expression of IL-5 (Rolfe et al., 1997). The expression characteristics of the IL-5 gene in HSB-2 cells are similar to those in normal T cells (Rolfe et al., 1997). These characteristics include the inducible expression of IL-5 by PMA/ionophore stimulation and repression of IL-5 expression by CsA and FK506 (Rolfe et al., 1997), but detailed transcriptional studies with this cell line have not been possible because of the lack of a sensitive transient expression assay for IL-5 reporter constructs. In this study the sensitive luciferase reporter gene was used to establish a transient transfection system in the human HSB-2 cell line. This system provides a new model for studying human IL-5 gene regulation and has allowed detailed transactivation and mutational studies to be carried out in the present work.

It is surprising that the flanking regions of the human and mouse genes share such limited homology. The region -80 to -20 is highly conserved (Fig.4a), but upstream and 3' regions give little indication of common elements that might be involved in the specific regulation of the gene. The conserved proximal region of IL-5 promoter contains potential binding sites for a number of transcription factors particularly AP-1, GATA and Ets/NFAT (Mizuta et al., 1988; Campbell et al., 1988; Zhang et al., 1997). The AP-1 and Ets/NFAT-binding sites together constitute an element called the consensus lymphokine element zero (CLE0) found in several cytokine genes including IL-3, IL-4, IL-5, and GM-CSF (Arai et al., 1990; Nimer, et al., 1990). This element plays a critical role in the regulation of the GM-CSF gene

(Miyatake et al., 1991; Nimer, et al., 1988; Wang et al., 1994). The CLE0 element also has a binding site for Oct (Thomas et al., 1999).

4.3.1. Activation of the human IL-5 gene in response to stimulation

The human IL-5 luciferase reporter gene was inducibly expressed 6-fold by stimulation with PMA/cAMP/ionophore in HSB-2 cells. The activation of the IL-5 promoter by PMA/cAMP was synergistically enhanced by ionophore, suggesting that expression of human IL-5 depends not only on PKA and PKC activation but also on an increase of intracellular Ca^{2+} in HSB-2 T cells. In D10W cells, IL-5 gene expression can be efficiently induced by PMA and cAMP alone (Chapter 3), indicating that slightly different signaling pathways are linked in induction of IL-5 gene expression in the two cell lines. Site-directed mutagenesis studies showed that inducible expression of the human IL-5 gene can be severely impaired by mutation of the binding sites for AP-1, Ets/NFAT and GATA in the proximal region of human IL-5 promoter, indicating that these binding sites are indispensible for IL-5 gene activation in HSB-2 cells as is the case for the mouse IL-5 promoter in D10W cells. It appears therefore that the core enhancers in the mouse and human IL-5 promoters are very similar.

4.3.2. Involvement of transcription factors in human IL-5 gene activation

Although the mutational studies showed that activation of human IL-5 gene expression in HSB-2 T cells required the AP-1, Ets/NFAT and GATA sites, transactivation studies were necessary to determine the transcription factors that

functioned at these sites. The -1.2khIL-5Luc construct is only induced 6-fold by PMA/cAMP/ionophore in HSB-2 cells but dramatic transactivations were achieved with expression constructs. AP-1, GATA-3 and Ets1 could transactivate the human IL-5 promoter 80-fold, 100-fold and 15-fold respectively, in the absence of stimulation. Thus in HSB-2 cells, the amount of AP-1 and GATA-3 in particular appear to be limiting the expression of the IL-5 promoter. The differences in the signaling pathways linked to IL-5 promoter induction in HSB-2 cells as compared to mouse D10W cells were also evident in the transactivation studies. In D10W cells, PMA was a very weak inducer relative to cAMP and the combination of PMA/cAMP enhanced IL-5 transactivation 2-10 times the levels achieved with cAMP alone. In contrast, in HSB-2 cells, PMA was 2-3 fold more effective as a stimulus than cAMP or ionophore alone. The combination of PMA/cAMP/ionophore increased transactivation 2-20 times that achieved with PMA. Despite the apparent differences in the pathways leading to gene activation, the transcription factors involved and the absolute dependence on the AP-1, Ets/NFAT and GATA sites were the same in the mouse and human systems. The transactivation data with the human IL-5 promoter strongly supported the findings with the mouse IL-5 promoter (Chapter 3) but was sufficiently different to give added verification of some aspects. For example, the evidence for the involvement of Ets1 in mouse IL-5 gene expression largely rested on the unique ability of Ets1 compared with Elf1, NFATp and NFATc to synergize with AP-1. In the human IL-5 promoter, Ets1 was much more active in transactivation than the other three factors potentially able to interact at the Ets/NFAT site. In addition, GATA-3 transactivation was very high with the human IL-5 promoter and strong evidence of cooperativity between GATA-3 and AP-1 and Ets1 was obtained in the transactivation studies. These results provide further support for the suggestion

(Chapter 3) that a higher order enhanceosome type complex may be involved in IL-5 gene regulation.

Although as discussed above some previous studies have not supported the involvement of the CLE0 element and adjacent GATA site in the proximal promoter region in human IL-5 expression, there is strong support for their role in expression of the mouse IL-5 gene (see Chapter 1). Given the strong conservation of the IL-5 proximal promoter region between human and mouse, it seems unlikely that the human IL-5 promoter would not be similarly regulated to the mouse promoter. The present work indicates that induction of the human IL-5 gene in HSB-2 cells involves cooperation between GATA-3, AP-1 and Ets1 binding in the proximal promoter region in an analogous way to induction of mouse IL-5 gene expression in the Th2 clone D10W. Support for a role for the GATA and CLE0 elements in human IL-5 gene expression has been provided by Yamagata et al., (1995, 1997). Thomas et al., (1999) also showed a role for CLE0 in the course of their studies on Oct involvement at the CLE0 site. A role for Oct was not examined in the present studies but it is possible that Oct cooperates with GATA-3, AP-1 and Ets1 in human IL-5 gene induction and further investigation of the role of Oct factors would be an interesting topic for future research.

Chapter 5 Role of Mitogen-activated Protein Kinase Signaling Pathways in the Regulation of Interleukin-5 Gene Expression

5.1. Introduction

Signal transduction via mitogen-activated protein (MAP) kinases plays a key role in a variety of cellular responses, including growth factor-induced proliferation, differentiation and cell death. Recently, several parallel MAP kinase signal transduction pathways have been defined in mammalian cells (Whitmarsh and Davis, 1996; Ip and Davis, 1998). These pathways include the extracellular signal related kinase (ERK) (Boulton et al., 1990, 1991), c-Jun N-terminal kinases (JNK, also known as SAPK) (Kyriakis, 1994), and p38 MAP kinase (Han et al., 1994; Lee et al., 1994b; Rouse et al., 1994). These MAP kinases are activated by phosphorylation on Thr and Tyr by dual-specificity MAP kinase kinases (Raingeaud et al., 1995). The MAP kinase groups are functionally independent and are implicated in different biological processes (Whitmarsh and Davis, 1996; Ip and Davis, 1998).

5.1.1. Cellular biology of MAP kinase pathway

The ERK is associated with proliferation and growth factors, while JNK and p38 are induced by stress responses and cytokines and can mediate differentiation and cell death. Several studies have described the participation of JNK and p38 MAP kinase in inflammation (Ip and Davis, 1998). It is likely that these kinases can also regulate the immune response. Three JNK genes have been identified in mammalian cells: JNK1, JNK2 and JNK3 (Ip and Davis, 1998). In addition, several isoforms

generated by alternative splicing of transcripts derived from these genes have been identified in humans (Gupta et al., 1996). JNK1 and JNK2 are widely expressed in several tissues, whereas JNK3 is selectively expressed in brain, testis and heart. The p38 MAP kinase gene was cloned following the purification of a 38 kDa protein that was phosphorylated on tyrosine in response to hyperosmolarity and endotoxic lipopolysaccharide. Human p38 MAP kinase homologues were identified as the targets of pyridinyl imidazole compounds, which inhibit the production of pro-inflammatory cytokines (IL-1, TNF-α) in stimulated macrophages (Han et al., 1994; Lee et al., 1994b; Rouse et al., 1994).

MAPKs are activated by other kinases functioning in a kinase cascade. Both JNK and p38 MAP kinase are activated by phosphorylation on threonine and tyrosine residues within the kinase subdomain VIII by dual-specificity MAP kinase kinases (MKK). The p38 MAP kinase is a selective target for pyridinyl imidazole drugs (Lee et al., 1994b). These drugs appear to act by inhibiting p38 MAP kinase activity through competition with ATP at the ATP-binding pocket (Tong et al., 1997; Wilson et al., 1997; Young et al., 1997). These compounds are candidate drugs for the treatment of arthritis, bone resorption and endotoxin shock (Griswold et al., 1988; Badger et al., 1996).

Two MAPK kinases, MKK4 and MKK7, have been found to be the primary activators of JNK (Derijard et al., 1995; Tournier et al., 1997). The p38 MAP kinase is activated by MKK3, MKK4 and MKK6 (Stein et al., 1996; Derijard et al., 1995; Han et al., 1996; Raingeaud et al., 1996;). The direct upstream kinase of ERK is MEK, which is regulated via phosphorylation by Raf (Daum et al., 1994). One of the JNK kinase activators (Fanger et al., 1997) is the mixed lineage kinase 3 (MLK3)

also known as the SH3 domain-containing proline-rich kinase (SPRK) (Rana et al., 1996).

JNK and p38 differ from ERK in that they are predominantly regulated by cellular stress inducers and proinflammatory cytokines (Kyriakis et al., 1994; Raingeaud et al., 1995). JNK is activated by SEK (SEK, also know as MKK4) as well as by the recently identified kinase MKK7 (Fanger et al., 1997). The activation of JNK is further controlled by a putative scaffold protein, JNK-interacting protein 1 (JIP-1), which binds to JNK and several other components of the JNK pathway (Whitmarsh et al., 1998). Overexpression of JIP-1 or the JNK-binding domain of JIP-1 leads to the cytoplasmic retention of JNK and the inhibition of JNK-dependent gene expression (Dickens et al., 1997) MAPK kinase 6 (MKK6) functions as an activating kinase for all known p38 isoforms (Han et al., 1996; Raingeaud et al, 1996; Enslen et al, 1998).

5.1.2. MAP kinase signal transduction during T cell activation

MAP kinase pathways play a critical role in the activation of T cells during the immune response. Several studies have shown the involvement of the ERK pathway in the positive selection of T cells in the thymus and in T-cell activation (Alberola-Ila et al., 1995; Swan et al., 1995). It has also been reported that JNK is activated during T-cell activation, where this molecule appears to play a role in integrating signals initiated at the TCR complex and the co-stimulatory molecule CD28 (Rincon and Flavell, 1994; Su et al., 1994). TCR ligation or TPA treatment is sufficient to maximally induce ERK activity, JNK and p38 activation requires a costimulatory signal such as CD28 ligand binding or ionomycin cotreatment, respectively (Dickens

et al., 1997; Su et al., 1994; Hoffmeyer et al., 1998). More recently, it has been shown that IL-2 and IL-7 activate both JNK and p38 kinases (Crawley et al., 1997).

A commonly used approach to determine the involvement of MAP kinase pathways is the use of inhibitors. Selective inhibitors of two of the MAP kinase pathways are available. PD98059 inhibits MEK1 and MEK2 activation with an IC_{50} of 5-10 μM and 50 μM respectively (Egerton et al., 1998) and is thus an inhibitor of the ERK pathway. SB203580 inhibits p38 MAP kinase (IC_{50}, 0.6μM). It is active on the p38α and β isoforms but not p38γ and δ (Hale et al., 1999). This is a potential difficulty for T cell studies as both the α and δ isoforms have been reported to be highly expressed in T cells (Jiang et al., 1997). Other approaches are the use of dominant negative expression constructs but in general these are often not highly effective (Hoffmeyer et al., 1999; Egerton et al., 1998). More effective have been positive activation constructs to induce individual MAP kinase pathway (Hoffmeyer et al., 1999).

The role of the three MAP kinase pathways in cytokine production by T cells has received comparatively little attention. In relation to the induction of IL-5 expression, stimulation mediated by the MAP kinase pathway could be achieved by phosphorylation of one or more of the relevant transcription factors within their activation domains or via the increased expression of one or more of the transcription factors or coactivators. For example, the basal levels of AP-1 proteins in T cells are generally low and T cell activation results in induction of Jun and Fos. JNK is believed to phosphorylate newly synthesized c-Jun and p38 MAP kinase can induce c-Fos and c-Jun levels (Karin, 1995)

SB203580 has been used to show a role for p38 MAP kinase in IL-5 expression in human T cells (Mori et al., 1999) and in the mouse T cell clone D10.G4.1 (Chen et al., 2000). Involvement of the ERK pathway in IL-5 expression in mouse T cells was demonstrated using PD98059 (Egerton et al., 1998). However, the relative importance of the MAP kinase pathways and their effectiveness in stimulating IL-5 expression in the absence of other stimuli has not yet been tested. This was investigated in the present work using activated expression constructs

5.2. Results

5.2.1. Activation of MAP kinase pathways in D10W cells

A transient transfection system was previously established to study the inducible activation of the mouse IL-5 gene in D10W cells (Chapter 3). Efficient induction of the -1170mIL-5Luc reporter gene was achieved by stimulation with PMA/cAMP. In the present work, this system was used to investigate the role of the ERK, JNK and p38 MAP kinase pathways in the induction of IL-5 transcription. Three expression plasmids previously shown to specifically stimulate the individual MAP kinase pathways were used for this study: (1) a constitutively active kinase mutant of Raf (Raf-BXB-CX) which serves as a specific ERK activator (Hoffmeyer et al., 1998); (2) MLK3, which when overexpressed results in a strong activation of JNK without affecting ERK and p38 MAP kinase activities; and (3) an activated mutant of MKK6 (MKK6E) which serves as a specific activator of p38 (Hoffmeyer et al., 1998). A p4 x AP-1/Ets promoter construct, which carries four copies of a combined AP-1/Ets binding from the polyoma virus enhancer in front of a luciferase gene, was used as a control. This enhancer element has previously been shown to be

responesive to constitutively active Raf in NIH3T3 cells (Bruder et al., 1992). It is also induced by MLK3 and MKK6E in the human T cell line A3.01 (Hoffmeyer et al., 1999). The results of the present work show that the transfection of Raf-BXB-CX also had a significant stimulatory effect on the transcriptional activity of the p4 x AP-1/Ets promoter in D10W cells. However, MLK3 induced the expression of this reporter construct to a lesser extent and MKK6E alone showed no significant effect on the promoter activity in D10W cells (Fig.5.1). The promoter activity induced by Raf or MLK3 in D10W cells was concentration-dependent (Fig.5.1). Combining Raf-BXB-CX with either MKK6E or MLK3 did not synergistically enhance the promoter activity (data not shown). However, the combination of Raf-BXB-CX, MKK6E and MLK3 resulted in over 30-fold induction of the p4 x AP-1/Ets construct which is double that achieved with Raf activation alone (Fig.5.2). Thus the p4 x AP-1/Ets promoter was a good indicator of ERK activation and therefore could be used to test the efficacy of the inhibitor PD98059 in D10W cells. A previous study using mouse T cells showed that a concentration of 100 μM PD98059 was required for near complete inhibition of the ERK pathway (Egerton et al., 1998). Therefore, the effect of concentrations of PD98059 of up to 120 μM were tested on Raf-stimulated p4 x AP-1/Ets expression. Surprisingly, there was no effect unless 20 μM SB203580 was also included (data not shown). The stimulation by Raf/MLK3/MKK6E was also significantly decreased by the same combination of PD98059/SB203580 (Fig.5.2).

5.2.2. Ability of MAP kinase pathways to induce IL-5 gene expression in D10W cells

The effect of activation of the MAP kinase pathways on IL-5 gene expression in D10W cells was then investigated. The -1170mIL-5Luc construct was

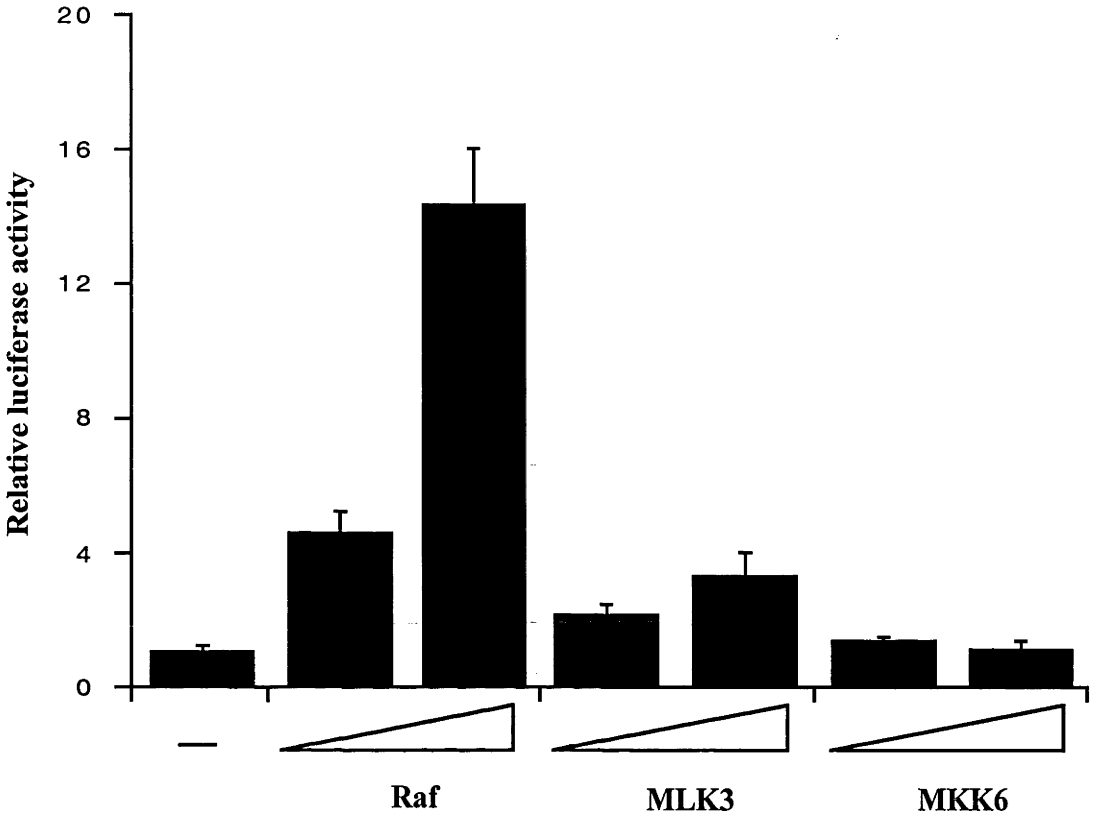


Fig. 5.1 Effect of the MAP kinase pathway activators Raf-BXB-CX, MLK3 and MKK6E on the induction of the p4 x AP-1/Ets promoter construct in D10W cells. The p4 x AP-1/Ets promoter construct was cotransfected alone with or 5 or 10μg of Raf-BXB-CX, MLK3, or MKK6E expression plasmids into D10W cells. At 20 hour posttransfection, cells were harvested, and luciferase activity was measured (see Materials and Methods). The results represent the average of at least three independent experiments.

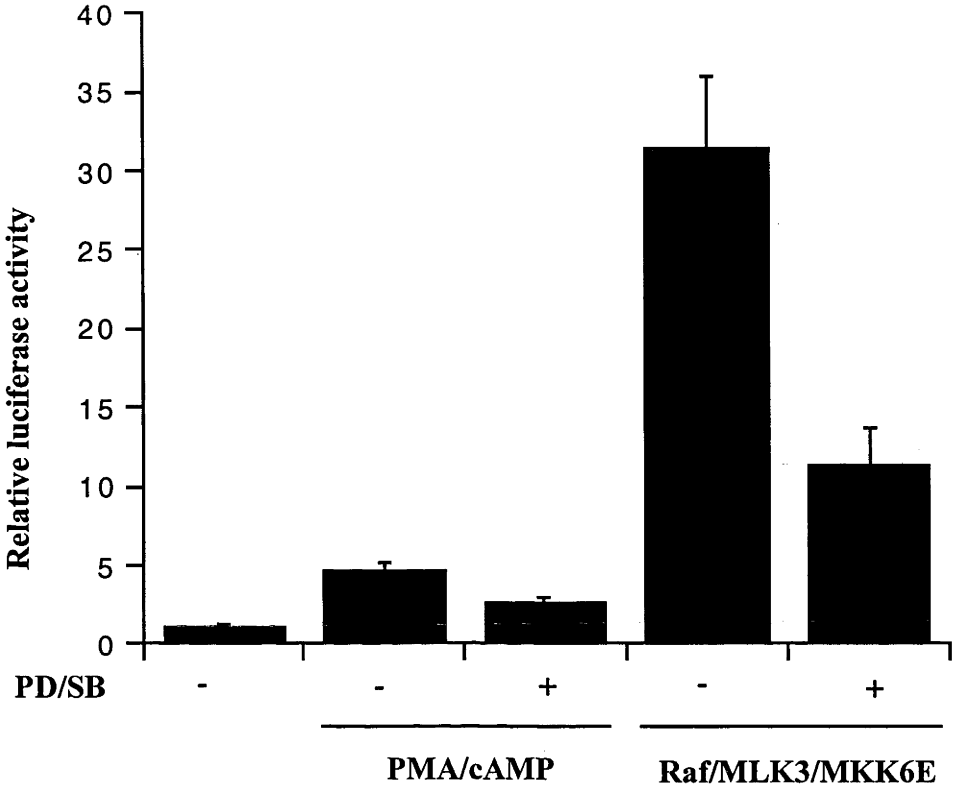


Fig. 5.2 Effect of MAP kinase inhibitors on the activation of the p4 x AP-1/Ets promoter construct in D10W cells. The p4 x AP-1/Ets promoter construct was cotransfected alone or with 5 μg of Raf-BXB-CX, MLK3 and MKK6E expression plasmids into D10W cells. Cells were left untreated (-) or treated with PMA/cAMP and PD98059 (120μM)/ SB203580 (20μM) (PD/SB) as indicated. Luciferase activity was measured (see Materials and Methods). The results represent the average of at least three independent experiments.

cotransfected with each of the MAP-kinase-stimulating expression constructs into D10W cells. As shown in Fig.5.3, expression of activated Raf gave up to 13-fold induction of IL-5 promoter activity in a dose-dependent manner. MLK3 gave up to 3-fold induction but MKK6E had smaller effect. The stimulation of all three kinase pathways together approximately doubled induction compared to stimulation by Raf alone (Fig.5.4). These results suggest that the activation of mouse IL-5 gene in D10W cells can be effectively induced by activation of the MEK/ERK pathway and to a lesser extent by stimulating the JNK pathway. Activation of the p38 MAP kinase pathway gave very slight induction of IL-5 expression. The results with the -1170mIL-5Luc construct were very similar to the effects on expression of the p4 x AP-1/Ets promoter suggesting that the stimulatory effects of the MAP kinase pathways may be exerted via the AP-1/Ets region of the IL-5 promoter.

It was of interest to see if PD98059, a specific inhibitor of the MEK/ERK pathway, could block the Raf-mediated stimulation of IL-5 transcription. In agreement with the results obtained with the p4 x AP-1/Ets promoter, Raf-induced IL-5 activity was not inhibited by PD98059 alone even at relatively high concentrations (120 μM) (Fig.5.5). However, Raf/MLK3/MKK6E-induced transcriptional activity of the IL-5 gene was significantly reduced by the combination of PD98059/SB203580 in D10W cells (Fig. 5.4).

The effects of PD98059 and SB203580 on induction of the IL-5 promoter in response to PMA/cAMP were also tested. The -1170mIL-5Luc reporter gene construct was transfected into D10W cells and the cells stimulated with PMA/cAMP in the presence and absence of PD98059 or SB203580. As shown in Fig.5.6, transcriptional activity of mouse IL-5 promoter was strongly induced in D10W cells

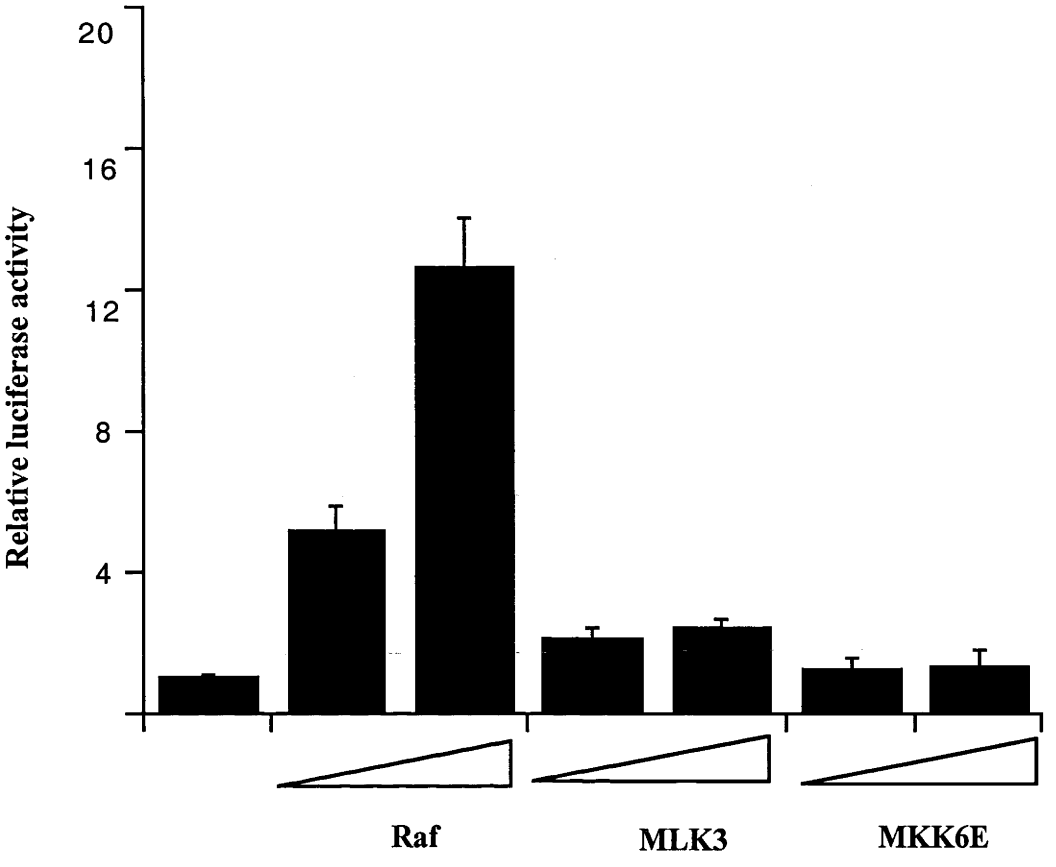


Fig. 5.3 Effect of expression of Raf-BXB-CX, MLK3, and MKK6E on the induction of the -1170mIL5Luc reporter construct in D10W cells. The -1170mIL5Luc construct was transfected alone or with 5 or 10μg of Raf-BXB-CX, MLK3 or MKK6E expression plasmids into D10W cells. At 20 hour posttransfection, cells were harvested and luciferase activity was measured (see Materials and Methods). The results represent the average of at least three independent experiments.

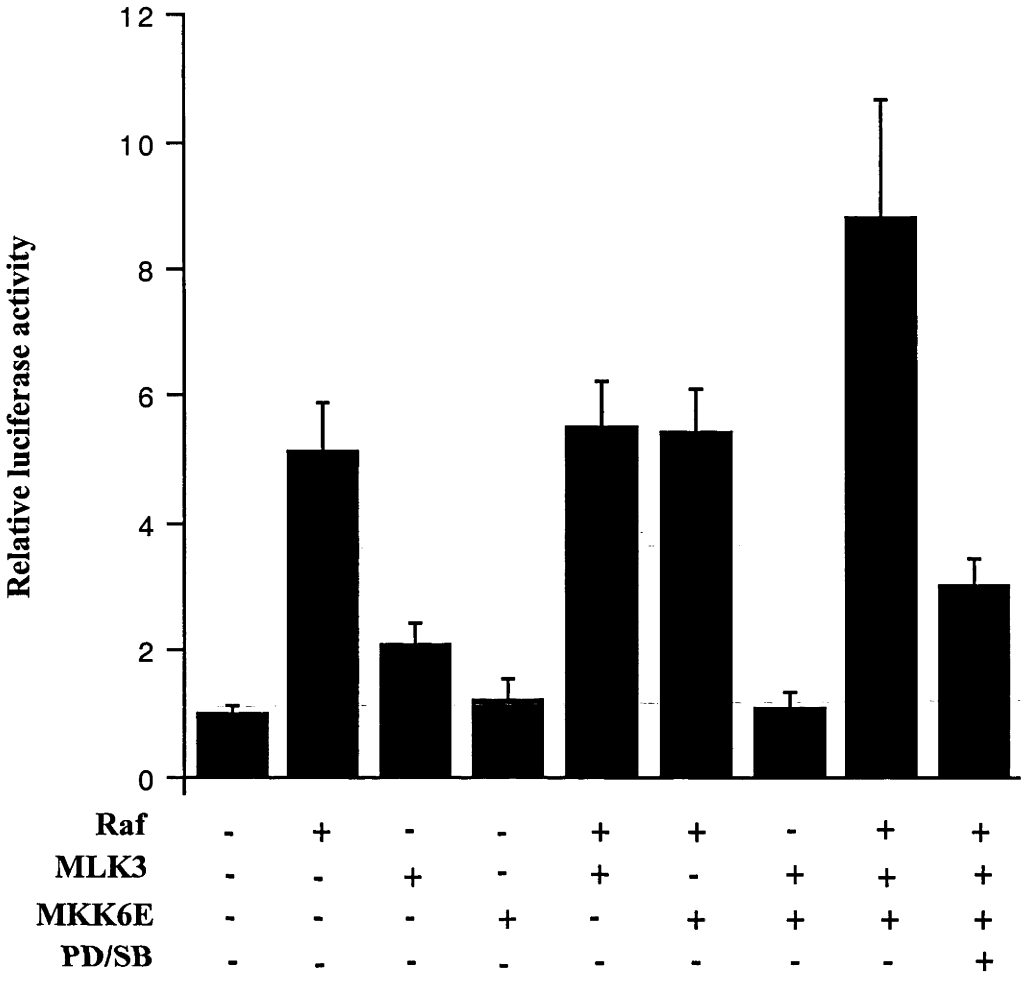


Fig. 5.4 Effect of expression of Raf-BXB-CX, MLK3 and MKK6E on the induction of the -1170mIL5Luc reporter construct in D10W cells. The -1170mIL5Luc construct was transfected alone or in combination with 5 μg of Raf-BXB-CX, MLK3, or MKK6E expression plasmids into D10W cells as indicated. Where inhibitors were used, cells were treated with 120 μM PD98059 (PD) and 20 μM SB203580 (SB) immediately after transfection. Luciferase activity was measured (see Materials and Methods). The results represent the average of at least three independent experiments.

following PMA/cAMP stimulation. Neither PD98059 nor SB203580 alone was able to block PMA/cAMP-induced IL-5 activity in D10W cells. However, in keeping with the above results, when relatively high concentrations of PD98059 and SB203580 were added together, a significant inhibitory effect was observed (Fig.5.6).

5.2.3. Enhanced activation of IL-5 transcription by each MAP kinase pathway in response to PMA and cAMP stimulation.

Since PMA/cAMP is a strong inducer of IL-5 gene expression, it was of interest to test the effects of PMA and cAMP in combination with stimulation of the ERK, JNK and p38 pathways on the induction of the IL-5 promoter. As shown in Fig.5.7, when transfected cells were treated with PMA and cAMP, very strong induction of the IL-5 promoter by Raf, MLK3 and MKK6E was observed. The highest stimulation was achieved with MKK6E (over 1300-fold). These results indicate that the MAP kinase pathways are being effectively stimulated by the expression constructs used and that stimulation of each MAP kinase pathway gives considerable stimulation of IL-5 expression. These high levels of stimulation require activation by PMA/cAMP and are very much greater than the stimulation obtained when all three MAP kinase pathways are stimulated together (Fig.5.6). This suggests that PMA/cAMP stimulation must activate an additional pathway or pathways important for IL-5 expression.

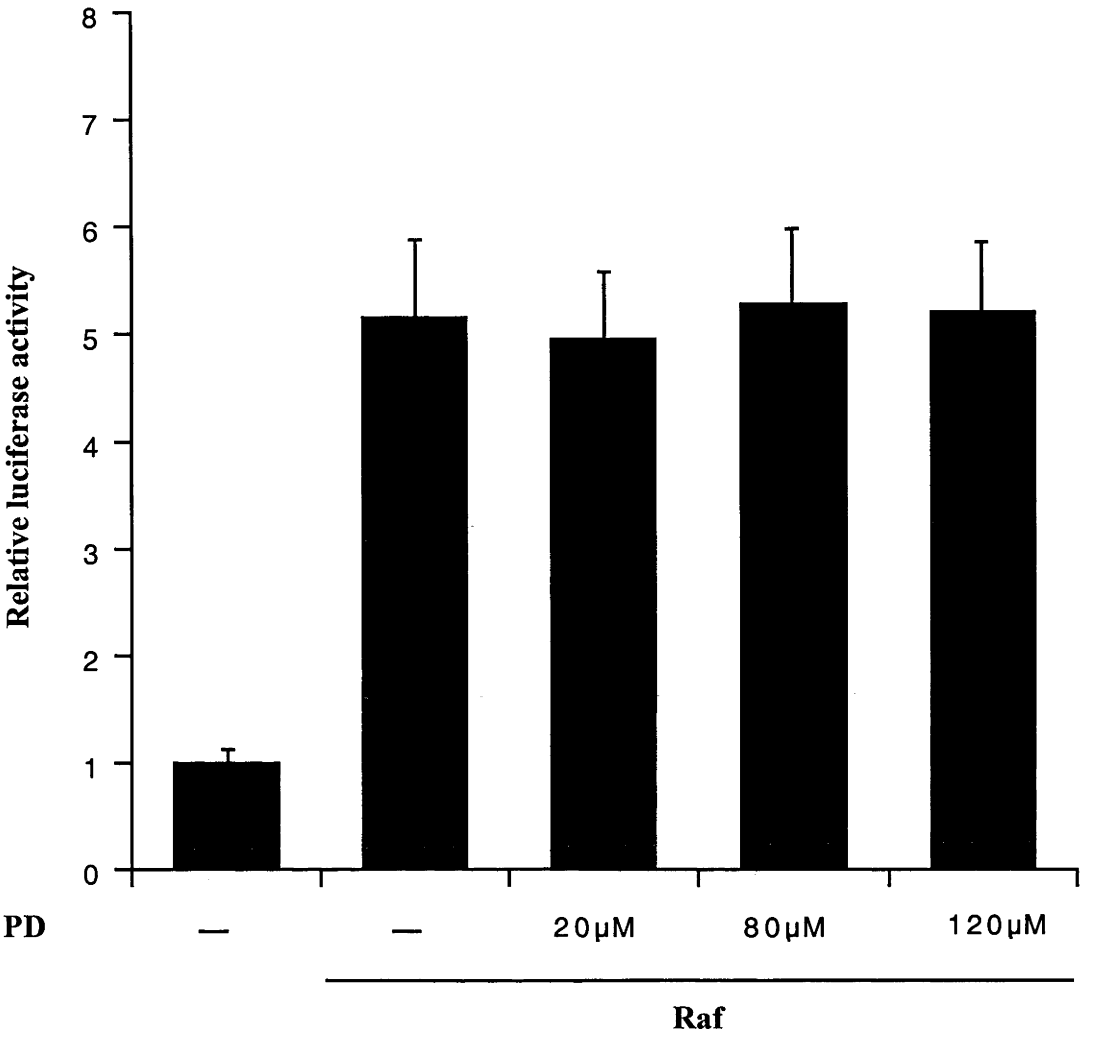


Fig. 5.5 Effect of different concentrations of PD98059 on the activation of the -1170mIL5Luc reporter construct induced by Raf in D10W cells. The -1170mIL5Luc construct was transfected alone or with 5 μg of the Raf-BXB-CX expression plasmid into D10W cells. Transfected cells were treated immediately after transfection with different amounts of PD98059 as indicated. Cells were harvested and luciferase activity was measured (see Materials and Methods). The results represent the average of at least three independent experiments.

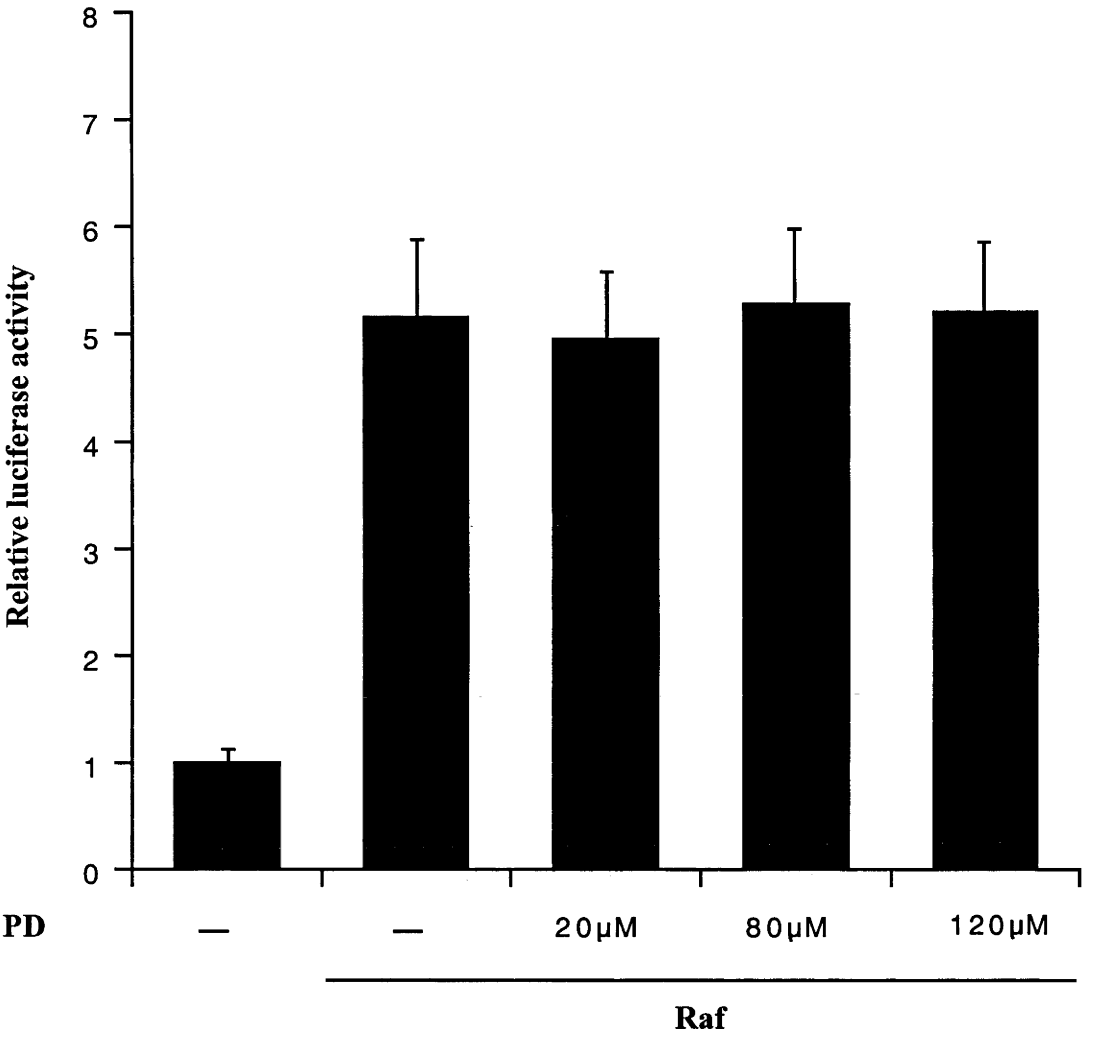


Fig. 5.5 Effect of different concentrations of PD98059 on the activation of the -1170mIL5Luc reporter construct induced by Raf in D10W cells. The -1170mIL5Luc construct was transfected alone or with 5 μg of the Raf-BXB-CX expression plasmid into D10W cells. Transfected cells were treated immediately after transfection with different amounts of PD98059 as indicated. Cells were harvested and luciferase activity was measured (see Materials and Methods). The results represent the average of at least three independent experiments.

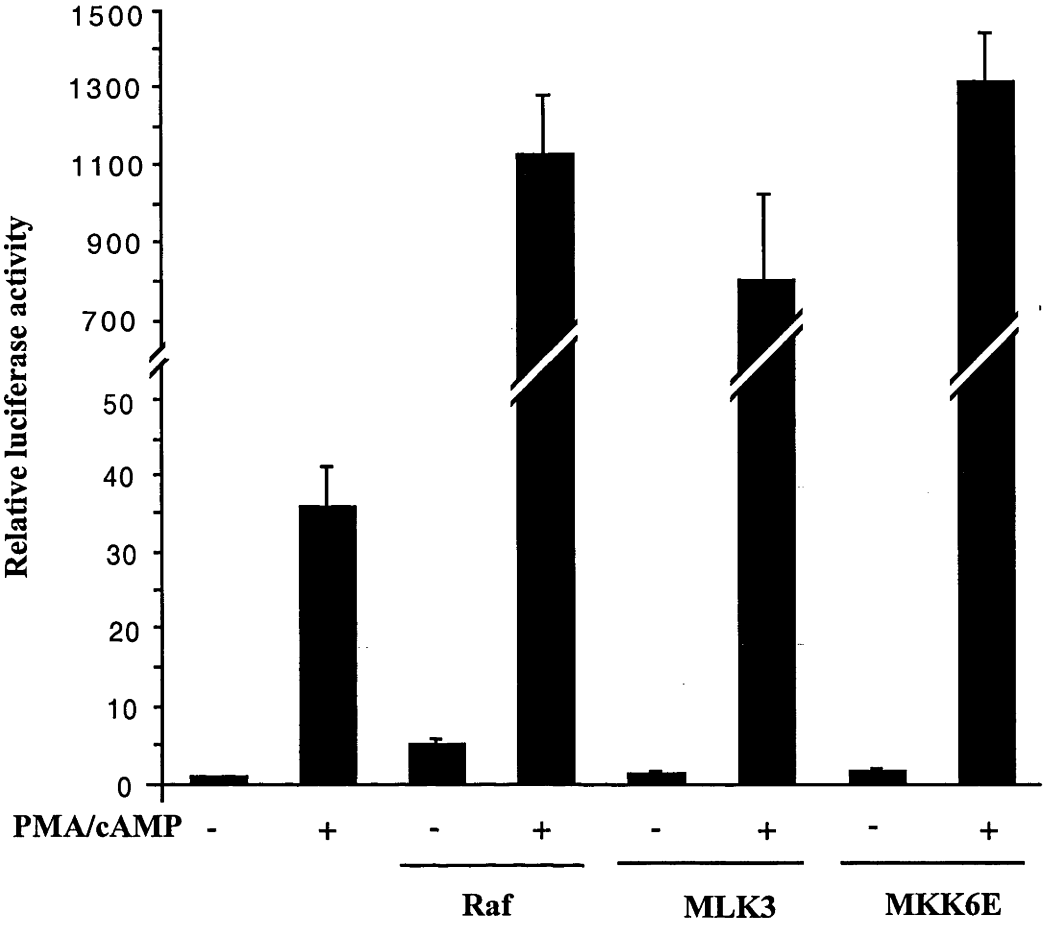


Fig. 5.7 Effect of expression of Raf-BXB-CX, MLK3 and MKK6E on induction of the -1170mIL5Luc reporter construct in response to PMA/cAMP in D10W cells. The -1170mIL5Luc construct was cotransfected with 5 μg of Raf-BXB-CX, MLK3, or MKK6E expression plasmids into D10W cells. At 20 hours posttransfection, cells were treated with PMA/cAMP for 9 hours. Luciferase activity was measured (see Materials and Methods). The results represent the average of at least three independent experiments.

5.2.4. Role of the putative transcription factor binding sites in MAP kinase-mediated IL-5 activity

In the work described in Chapter 3, it was shown that the AP-1, Ets and GATA elements in the proximal promoter region were essential for IL-5 expression. To determine whether the stimulation of IL-5 expression mediated by activation of the MAP kinase pathways was dependent on these binding sites, wild-type and mutant -1170mIL-5Luc constructs were transfected together with expression vectors for Raf-BXB-CX, MLK3 or MKK6E into D10W cells. Mutation of each of the three transcription factor binding sites greatly reduced the induction of IL-5 expression mediated by each MAP kinase pathway (Fig.5.8).

5.2.5. Effect of MAP kinases on distal and proximal IL-5 promoter activity in D10W cells

To study whether the effect of each MAP kinase pathway on IL-5 activation in response to PMA/cAMP treatment also requires upstream promoter elements, the proximal promoter construct -88mIL-5Luc was compared with -1170mIL-5Luc. These constructs were transfected together with expression constructs for Raf, MLK3 or MKK6E into D10W cells. The transfected cells were treated with or without PMA/cAMP. The results showed that the -88mIL-5Luc reporter gene construct was also strongly stimulated by each expression construct in response to PMA/cAMP stimulation (Fig.5.9). It is therefore reasonable to conclude that the major effects of the MAP kinase pathways involve the proximal promoter region to -88 and that there is no obligatory involvement of elements further upstream.

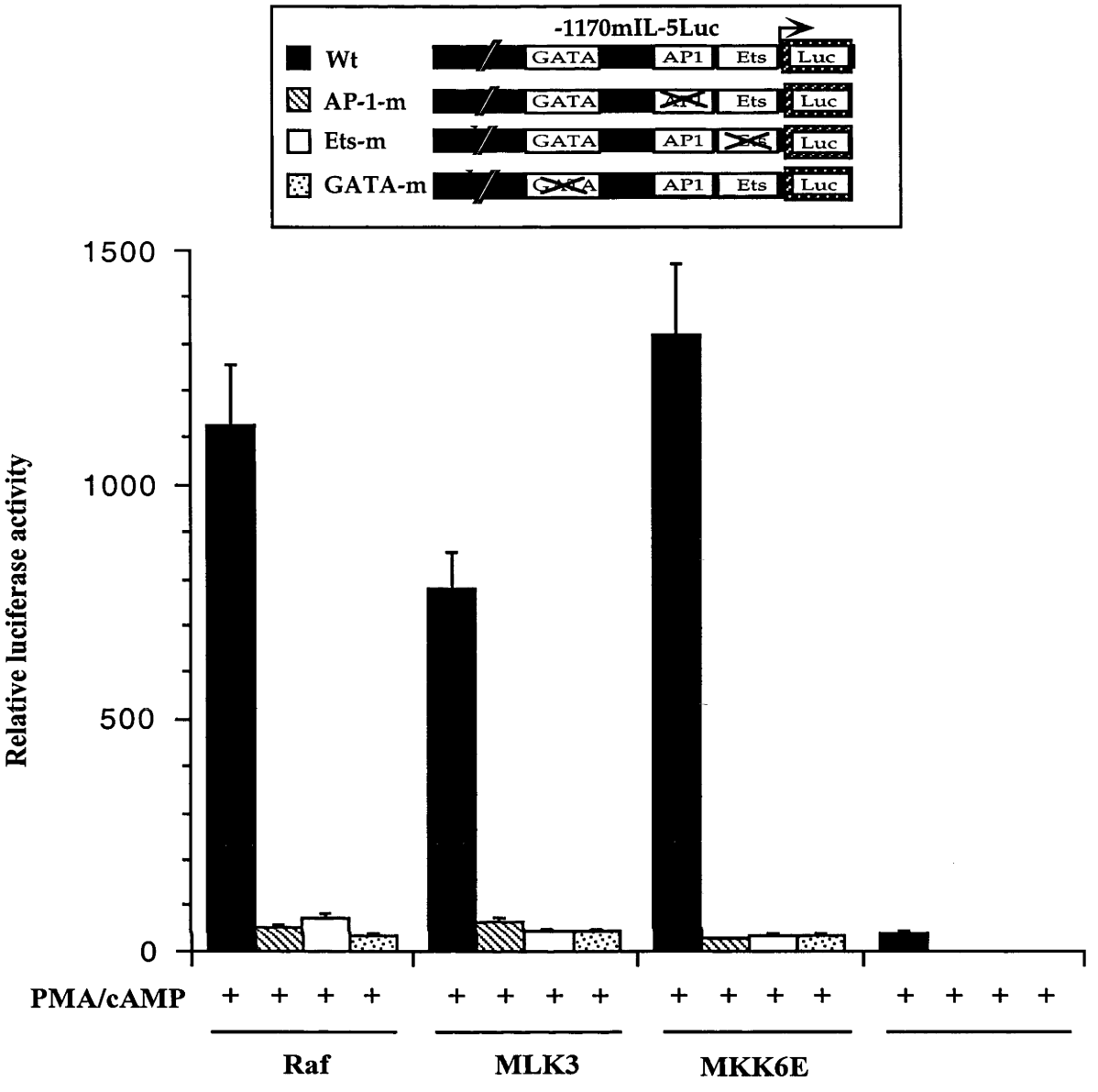


Fig.5.8 Effect of Mutation of the transcription factor binding sites on Raf-, MLK3- or MKK6E-mediated IL-5 transcription activity in D10W cells. The structures of the mutant mouse IL-5 promoter constructs are shown schematically. The indicated mutant constructs were cotransfected with expression plasmids for Raf-BXB-CX, MLK3 and MKK6E into D10W cells. Luciferase activity was measured (see Materials and Methods). Results represent the average of three independent experiments.

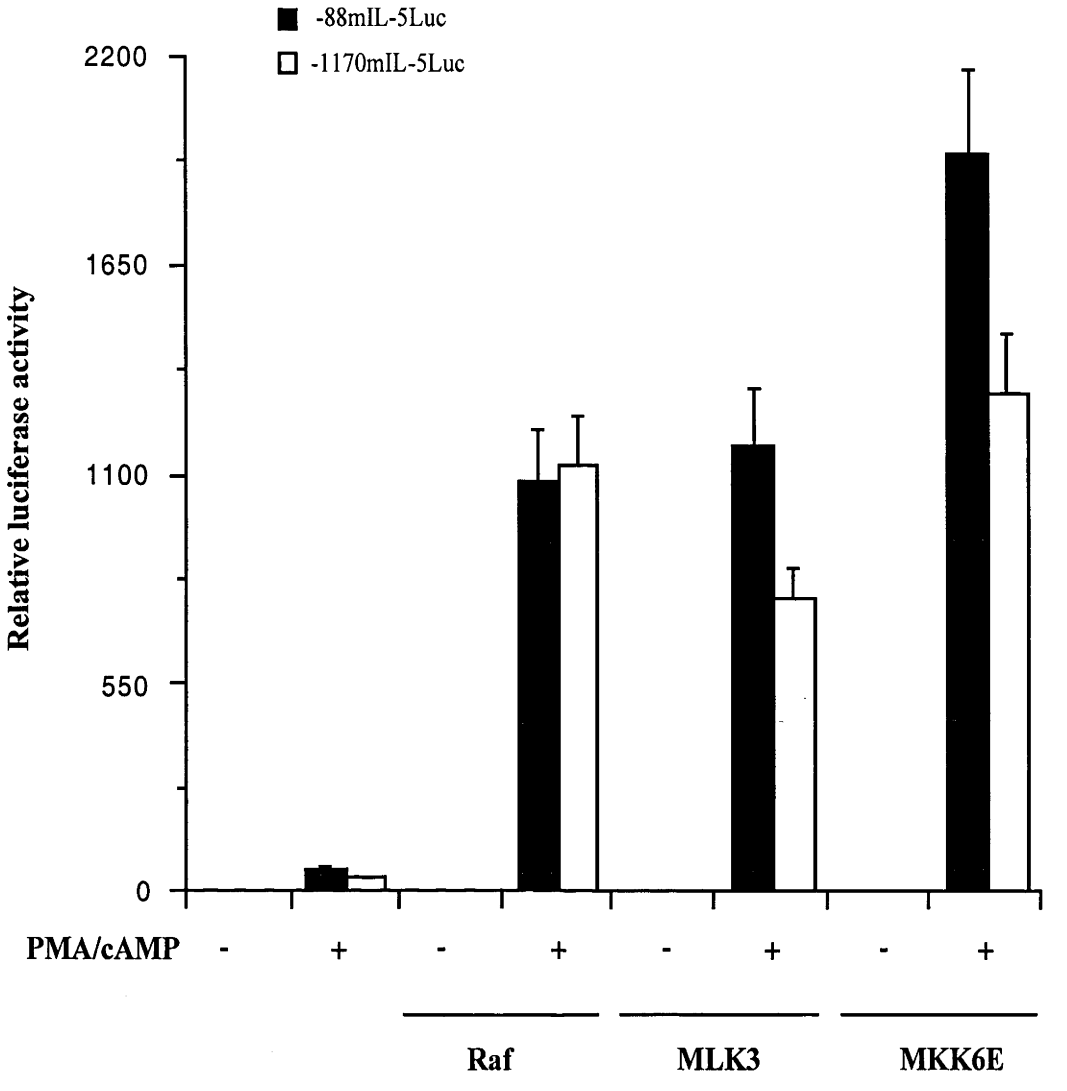


Fig. 5.9 A reporter construct carrying only the proximal promoter region of the IL-5 gene shows strong stimulation by expression of Raf-BXB-CX, MLK3, or MKK6E in response to PMA/cAMP stimulation in D10W cells. The indicated promoter constructs were cotransfected with either Raf-BXB-CX, MLK3, or MKK6E expression plasmids into D10W cells. At 20 hours posttransfection, cells were either left untreated (-) or treated (+) with PMA/cAMP. Luciferase activity was measured (see Materials and Methods). The results represent the average of at least three independent experiments.

5.3. Discussion

In this Chapter, the involvement of MAP kinase pathways in IL-5 promoter activation in D10W cells was studied. Three expression constructs were used to specifically activate the ERK, JNK or p38 MAP kinase pathways. A reporter system (p4 x AP-1/Ets) shown in a human T cell line to be responsive to activation of each of the MAP kinase pathways was used as a control. This reporter had the additional advantage of being closely related to the IL-5 promoter which also carries an essential AP-1/Ets site. Raf-BXB-CX, a constitutively active kinase mutant, which lacks the N-terminal negative regulatory domain and contains the C-terminal membrane targeting 17 amino acids of Ki-Ras fused to the kinase domain of c-Raf (Hoffmeyer et al., 1998) was used to activate the ERK pathway. Overexpression of MLK3 was used to activate JNK without affecting ERK and p38 activities. MKK6E which is a constitutively active mutant of MKK6 with two serines involved in the activation of the kinase replaced by glutamic acid (Raingeaud et al., 1996) was used to activate p38. The expression constructs used have been verified by others as specific activators of ERK, JNK, and p38 (Hoffmeyer et al., 1998, 1999). Recent inhibitor studies have indicated involvement of the ERK and p38 MAP kinase pathway in IL-5 expression (Egerton et al., 1998; Mori et al., 1999; Chen et al., 2000). The present work has shown for the first time that activation of the ERK pathway, in the absence of other stimulation, is sufficient to induce IL-5 transcription (Fig.5.3). The activation of JNK also induced IL-5 expression but to a lesser extent. The effects of the -1170mIL-5Luc construct were closely similar to the induction of the p4X AP-1/Ets promoter (Fig.5.1) which was used as a control. This suggests that the stimulatory effects of the MAP kinase pathways are being mediated via the AP-1/Ets element in

the IL-5 promoter. This could involve elevation of AP-1 levels as the studies described in Chapter 3 demonstrated that overexpression of AP-1 is sufficient to induce IL-5 transcription and/or activation of AP-1 and Ets1 by phosphorylation. c-Jun is known to be phosphorylated by JNK (Karin, 1995) and Ets1 can be phosphorylated by ERK (Rabault et al., 1996). Ras is know to activate Ets1 and Ets2 and to induce transcription through AP-1/Ets composite element (Wasylyk et al., 1997; Patton et al., 1998).

The ability of each of the three MAP kinases to strongly stimulate IL-5 expression was shown when transfected cells were treated with PMA/cAMP. The Raf-induced IL-5 activation was increased over 1000-fold with PMA/cAMP stimulation and overproduction of MLK3 and MKK6E in cells stimulated with PMA/cAMP resulted in 800-fold and 1300-fold induction of the IL-5 activity over basal levels. The impressive stimulation by the MAP kinases were dependent on the GATA, AP-1 and Ets sites in the proximal promoter region and did not require the upstream promoter region of the IL-5 gene. The results with IL-5 are somewhat similar to the role of MAP kinases in T cell expression of TNFα (Hoffmeyer et al., 1999) where activation of each pathway is sufficient to induce expression and it appears that the three MAP kinase pathways cooperate to induce TNFα expression (Hoffmeyer et al., 1999). In the case of IL-5 expressionin D10W cells such cooperation could also occur. A role for MEK and p38 in IL-5 expression are supported by other studies (Egerton et al., 1998; Mori et al., 1999; Chen et al., 2000). cAMP can stimulated activation of p38 kinase in D10.G4.1 which can result in increased phosphorylation of GATA-3 and increased expression of IL-5 (Chen et al., 2000). Currently, there is no additional evidence supporting a role for JNK in IL-5

expression although its well studied role in AP-1 activation (Karin, 1995) makes its involvement likely. It is clear from the present work, however, that activation of the three MAP kinase pathways alone is not sufficient to achieve optimal transcription of IL-5. PMA/cAMP stimulation provides an important additional signal or signals.

The MAP kinase inhibitor results in these studies were unexpected. PD98059 would normally be expected to inhibit ERK activation on its own without the requirement for SB203580. One possible explanation could be that SB203580 has been shown to be a specific inhibitor of p38 MAP kinase only at low concentrations and to additionally inhibit the ERK pathway at the concentration used in this work (20 μM). Perhaps both PD98059 and SB203580 are required to effectively inhibit ERK in D10W cells. There was not sufficient time to investigate this further by direct measurements of MAP kinase inhibition.

Chapter 6 General Discussion

6.1. Discussion

The present work has concentrated on functional studies of the transcription factors involved in binding to the proximal region of the IL-5 promoter and in inducing gene expression. This aspect has not received much attention in previous studies. In particular, it has been unclear whether Ets or NFAT family members function together with AP-1 at the CLE0 site. Transient transfection systems have been established in mouse and human cell lines which are good models for IL-5 expression in normal T lymphocytes. D10W cells are a closer model of mouse Th2 lymphocytes than the widely used mouse thymoma EL-4 and HSB-2 cells provide a generally available system for human IL-5 expression studies.

The importance of the proximal promoter region for IL-5 gene induction was supported by previous studies in this laboratory of the mouse IL-5 gene in a normal chromatin environment (Bourke et al., 1995; Tan, 1998; Young et al., 1999). The results indicate that the proximal promoter region to -88 is responsible for Th2-specific expression and gene induction (Tan, 1998; Young et al., 1999). However, there appears to be elements further upstream which are also important in IL-5 expression such as in the repression by dexamethasone (Tan, 1998; Young et al., 1999). For this reason, in the present work, the -1170mIL-5Luc and -1.2khIL-5Luc reporter constructs were used which carry approximately 1200 bp of upstream sequence and, where required, mutations were made in elements in the proximal promoter region of these constructs. An obligatory role for the GATA, AP-1 and Ets/NFAT sites in the proximal IL-5 promoter in human and mouse was

demonstrated. Comparison with a construct truncated to -88 allowed verification that there was no obligatory involvement of upstream elements in mouse IL-5 gene induction and transactivation.

The mouse and human transactivation studies provide complementary data that supports an obligatory role of the proximal AP-1, Ets/NFAT and GATA sites in promoter induction and indicate cooperative interactions between AP-1 (c-Fos/c-Jun), Ets1 and GATA-3. Very high levels of transactivation and synergism were achieved in some cases in these studies but mutation of the GATA, AP-1 and Ets/NFAT sites showed that these sites played a critical role in the transactivations observed.

The signaling pathways linked to IL-5 gene induction were somewhat different in D10W and HSB-2 cells. In D10W, PMA alone was a very weak inducer of IL-5 expression in comparison to cAMP but the two agents strongly synergized in gene induction. In HSB-2 cells, PMA alone was more effective than cAMP or Ca^{2+} ionophore as an inducer but the three stimuli synergized to give the most effective IL-5 induction. In both cells, elevation of AP-1 levels using expression constructs for c-Fos and c-Jun resulted in strong induction of IL-5 expression in the absence of stimulation. DNA binding studies have shown that AP-1 binding to the CLE0 element of the mouse IL-5 promoter is strongly induced by stimulation (Siegel et al., 1995; Karlen et al., 1996; Zhang et al., 1997; Tan, 1998; Young et al., 1999) and the transactivation results suggest that elevation of AP-1 levels is important in normal IL-5 gene induction. Gene activation involving upregulation of AP-1 has also been reported in other cases. Stimulation of expression of AP-1 accompanies the activation of the AP-1 transcriptional complex by insulin (Griffiths et al., 1998) and the cAMP

response of the dopamine-β hydroxylase gene is mediated via AP-1 (Swanson et al., 1998).

In HSB-2 cells, elevation of GATA-3 levels also resulted in strong IL-5 gene induction without stimulation. Thus in HSB-2 cells, in contrast to D10W cells, the levels of GATA-3 in unstimulated cells appear to limit IL-5 expression. GATA-3 levels may be elevated to some extent by stimulation as part of the IL-5 induction process. On the other hand, low GATA-3 levels may explain why the IL-5 gene is less effectively induced in HSB-2 than in D10W. Comparison of the effectiveness of GATA-3 and GATA-4 in both mouse and human IL-5 induction showed that they were of comparable activity. Since GATA-3 is normally expressed in T cells, it would be involved in IL-5 expression in normal T lymphocytes.

The transactivation studies also showed strong cooperative effects between AP-1, Ets1 and GATA-3. In D10W cells there was strong synergy between Ets1 and AP-1 and in HSB-2 strong synergistic effects were seen between all three factors. This suggests that the three factors may be part of a higher order complex analogous to the enhanceosome of the IFN-β promoter (Yie et al., 1997). The preliminary indication of binding sites and a role for HMGI(Y) in the proximal mouse IL-5 promoter suggest that this architectural transcription factor could be also be involved. No indications are yet available as to possible coactivators involved in IL-5 gene expression.

The unique ability of Ets1 to synergize with AP-1 in D10W cells and with AP-1 and GATA-3 in HSB-2 cells provides evidence that this factor, rather than Elf1, NFATc or NFATp, functions at the Ets/NFAT site in the CLE0 element. Recombinant Ets1 bound to this site but only weakly in EMSA experiments

(Fig.3.12). It is possible that *in vivo* other proteins in the postulated higher order complex promote Ets1 binding. A recent study showed that the proteins involved in formation of the constitutive complexes with the CLE0 element in EL-4 cells belong to the octamer transcription factor family and this finding was confirmed in primary mouse T cells (Salerno et al., 2001). The functional activity of octamer factors binding to the mouse IL-5 CLE0 element was supported by ectopic expression of Oct1 and Oct2 with a mouse IL-5 reporter gene construct. Both octamer factors had small positive effects on mouse IL-5 reporter gene activity with Oct1 having the greatest impact (Salerno et al., 2001). Oct2 binding to a region between -94 to -64 in the IL-2 promoter has been shown to have a positive effect on the expression of this gene. The close association between Oct2 and AP-1 in this segment of the gene was crucial for the optimal induction of IL-2 promoter in T cells (Kamp et al., 1990; Pfeuffer et al., 1994). The association between Oct1, Oct2, AP-1 and NFAT proteins in IL-4 promoter region has also been shown to be essential for the overall activity of this promoter in T cells (Li-Weber et al., 1998). The octamer transcription factors have also been reported to bind to the human IL-5 CLE0 element (Thomas et al., 1999). It would be of interest to establish if Oct1 or Oct2 could cooperate with AP-1, Ets1 or GATA-3 in the transient transfection systems described in this work.

The MAP kinase studies described in Chapter 5 support the possibility that the three MAP kinase pathways cooperate in IL-5 gene induction. Activation of the ERK pathway and to a lesser extent the JNK pathway was sufficient to induce IL-5 expression without stimulation. This activation appeared to be mediated via the CLE0 element as an analogous p4 x AP-1/Ets enhancer construct was similarly induced. Simultaneous activation of all three MAP kinase pathways did not give maximal IL-5

expression in the absence of stimulation. However, when D10W cells were stimulated with PMA/cAMP, activation of each MAP kinase pathway resulted in very high expression levels indicating that activation of another singaling pathway (or pathways), in addition to the MAP kinase pathways, is required for optimal IL-5 expression. The impressive MAP kinase stimulations were also observed with a construct truncated to -88 and were dependent on the proximal GATA, AP-1 and Ets/NFAT sites indicating that they were mediated through the proximal promoter region.

Overall, the work described in this thesis supports the concept that the proximal promoter region of the mouse and human IL-5 genes is the major element regulating inducible gene expression. Th2 specificity appears to be largely due to the essential involvement of GATA-3, a Th2-specific transcription factor (Zhang et al., 1997, 1998, 1999; Zheng and Flavell, 1997) but obligatory involvement of AP-1 and Ets1 is also required. The three MAP kinase pathways may cooperate in gene induction via the proximal promoter elements in stimulated cells. ERK activation alone is sufficient to induce gene expression via the AP-1/Ets site. Upregulation of AP-1 is an important part of normal gene induction and is sufficient to induce the IL-5 gene. Activation of the three transcription factors by phosphorylation is probably also involved. The impressive synergistic interactions between AP-1, Ets1 and GATA-3 suggest a higher order complex possibly involving HMGI(Y) and one or more coactivators whose activity could be modulated by regulatory elements elsewhere in the IL-5 gene.

7. References

Agarwal S, Rao A (1998) Modulation of chromatin structure regulates cytokine gene expression during T cell differentiation. Immunity 9:765-775.

Agarwal S, Viola JP, Rao A (1999) Chromatin-based regulatory mechanisms governing cytokine gene transcription. J Allergy Clin Immunol 103:990-999.

Alberola-Ila J, Forbush KA, Seger R, Krebs EG, Perlmutter RM (1995) Selective requirement for MAP kinase activation in thymocyte differentiation. Nature 373:620-623.

Angel P, Karin M (1991) The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. Biochim Biophys Acta 1072:129-157.

Arai KI, Lee F, Miyajima A, Miyatake S, Arai N, Yokota T (1990) Cytokines: coordinators of immune and inflammatory responses. Annu Rev Biochem 59:783-836.

Azuma C, Tanabe T, Konishi M, Kinashi T, Noma T, Matsuda F, Yaoita Y, Takatsu K, Hammarstrom L, Smith CI, et al. (1986) Cloning of cDNA for human T-cell replacing factor (interleukin-5) and comparison with the murine homologue. Nucleic Acids Res 14:9149-9158.

Badger AM, Bradbeer JN, Votta B, Lee JC, Adams JL, Griswold DE (1996) Pharmacological profile of SB 203580, a selective inhibitor of cytokine suppressive binding protein/p38 kinase, in animal models of arthritis, bone resorption, endotoxin shock and immune function. J Pharmacol Exp Ther 279:1453-1461.

Bassuk AG, Leiden JM (1995) A direct physical association between ETS and AP-1 transcription factors in normal human T cells. Immunity 3:223-237.

Basuyaux JP, Ferreira E, Stehelin D, Buttice G (1997) The Ets transcription factors interact with each other and with the c- Fos/c-Jun complex via distinct protein domains in a DNA-dependent and - independent manner. J Biol Chem 272:26188-26195.

Baxevanis AD, Vinson CR (1993) Interactions of coiled coils in transcription factors: where is the specificity? Curr Opin Genet Dev 3:278-285.

Bazan JF (1990) Shared architecture of hormone binding domains in type I and II interferon receptors. Cell 61:753-754.

Beals CR, Clipstone NA, Ho SN, Crabtree GR (1997) Nuclear localization of NF-ATc by a calcineurin-dependent, cyclosporin- sensitive intramolecular interaction. Genes Dev 11:824-834.

Ben-David Y, Giddens EB, Letwin K, Bernstein A (1991) Erythroleukemia induction by Friend murine leukemia virus: insertional activation of a new member of the ets gene family, Fli-1, closely linked to c-ets-1. Genes Dev 5:908-918.

Bergelson S, Daniel V (1994) Cooperative interaction between Ets and AP-1 transcription factors regulates induction of glutathione S-transferase Ya gene expression. Biochem Biophys Res Commun 200:290-297.

Bert AG, Burrows J, Osborne CS, Cockerill PN (2000) Generation of an improved luciferase reporter gene plasmid that employs a novel mechanism for high-copy replication. Plasmid 44:173-182.

Betts CJ, Else KJ (1999) Mast cells, eosinophils and antibody-mediated cellular cytotoxicity are not critical in resistance to Trichuris muris. Parasite Immunol 21:45-52.

Bielinska M, Wilson DB (1995) Regulation of J6 gene expression by transcription factor GATA-4. Biochem J 307:183-189.

Blumenthal SG, Aichele G, Wirth T, Czernilofsky AP, Nordheim A, Dittmer J (1999) Regulation of the human interleukin-5 promoter by Ets transcription factors. Ets1 and Ets2, but not Elf-1, cooperate with GATA3 and HTLV-I Tax1. J Biol Chem 274:12910-12916.

Bohmann D, Bos TJ, Admon A, Nishimura T, Vogt PK, Tjian R (1987) Human proto-oncogene c-jun encodes a DNA binding protein with structural and functional properties of transcription factor AP-1. Science 238:1386-1392.

Boulton TG, Gregory JS, Cobb MH (1991) Purification and properties of extracellular signal-regulated kinase 1, an insulin-stimulated microtubule-associated protein 2 kinase. Biochemistry 30:278-286.

Boulton TG, Yancopoulos GD, Gregory JS, Slaughter C, Moomaw C, Hsu J, Cobb MH (1990) An insulin-stimulated protein kinase similar to yeast kinases involved in cell cycle control. Science 249:64-67.

Boulukos KE, Pognonec P, Begue A, Galibert F, Gesquiere JC, Stehelin D, Ghysdael J (1988) Identification in chickens of an evolutionarily conserved cellular ets- 2 gene (c-ets-2) encoding nuclear proteins related to the products of the c-ets proto-oncogene. Embo J 7:697-705.

Bourke PF, van Leeuwen BH, Campbell HD, Young IG (1995) Localization of the inducible enhancer in the mouse interleukin-5 gene that is responsive to T-cell receptor stimulation. Blood 85:2069-2077.

Boyce JA, Friend D, Matsumoto R, Austen KF, Owen WF (1995) Differentiation in vitro of hybrid eosinophil/basophil granulocytes: autocrine function of an eosinophil developmental intermediate. J Exp Med 182:49-57.

Bradford AP, Conrad KE, Wasylyk C, Wasylyk B, Gutierrez-Hartmann A (1995) Functional interaction of c-Ets-1 and GHF-1/Pit-1 mediates Ras activation of pituitary-specific gene expression: mapping of the essential c-Ets-1 domain. Mol Cell Biol 15:2849-2857.

Braun MY, Desalle F, Le Moine A, Pretolani M, Matthys P, Kiss R, Goldman M (2000) IL-5 and eosinophils mediate the rejection of fully histoincompatible vascularized cardiac allografts: regulatory role of alloreactive CD8(+) T lymphocytes and IFN-gamma. Eur J Immunol 30:1290-1296.

Broide DH, Paine MM, Firestein GS (1992) Eosinophils express interleukin 5 and granulocyte macrophage-colony- stimulating factor mRNA at sites of allergic inflammation in asthmatics. J Clin Invest 90:1414-1424.

Bruder JT, Heidecker G, Rapp UR (1992) Serum-, TPA-, and Ras-induced expression from Ap-1/Ets-driven promoters requires Raf-1 kinase. Genes Dev 6:545-556.

Brusselle GG, Kips JC, Tavernier JH, van der Heyden JG, Cuvelier CA, Pauwels RA, Bluethmann H (1994) Attenuation of allergic airway inflammation in IL-4 deficient mice. Clin Exp Allergy 24:73-80.

Burgess AW (1988) Cell surface heparan sulphate implicated in haemopoietic growth factor signalling. Immunol Today 9:267-268.

Burtis KC, Thummel CS, Jones CW, Karim FD, Hogness DS (1990) The Drosophila 74EF early puff contains E74, a complex ecdysone- inducible gene that encodes two ets-related proteins. Cell 61:85-99.

Bustin M, Reeves R (1996) High-mobility-group chromosomal proteins: architectural components that facilitate chromatin function. Prog Nucleic Acid Res Mol Biol 54:35-100.

Butterworth AE (1984) Cell-mediated damage to helminths. Adv Parasitol 23:143-235.

Campbell HD, Tucker WQ, Hort Y, Martinson ME, Mayo G, Clutterbuck EJ, Sanderson CJ, Young IG (1987) Molecular cloning, nucleotide sequence, and expression of the gene encoding human eosinophil differentiation factor (interleukin 5). Proc Natl Acad Sci U S A 84:6629-6633.

Campbell HD, Sanderson CJ, Wang Y, Hort Y, Martinson ME, Tucker WQ, Stellwagen A, Strath M, Young IG (1988) Isolation, structure and expression of cDNA and genomic clones for murine eosinophil differentiation factor. Comparison with other eosinophilopoietic lymphokines and identity with interleukin-5. Eur J Biochem 174:345-352.

Carr PD, Gustin SE, Church AP, Murphy JM, Ford SC, Mann DA, Woltring DM, Walker I, Ollis DL, Young IG (2001) Structure of the complete extracellular domain of the common beta subunit of the human GM-CSF, IL-3, and IL-5 receptors reveals a novel dimer configuration. Cell 104:291-300.

Chaiken IM, Williams WV (1996) Identifying structure-function relationships in four-helix bundle cytokines: towards de novo mimetics design. Trends Biotechnol 14:369-375.

Chand N, Harrison JE, Rooney S, Pillar J, Jakubicki R, Nolan K, Diamantis W, Sofia RD (1992) Anti-IL-5 monoclonal antibody inhibits allergic late phase bronchial eosinophilia in guinea pigs: a therapeutic approach. Eur J Pharmacol 211:121-123.

Chandrasekharappa SC, Rebelsky MS, Firak TA, Le Beau MM, Westbrook CA (1990) A long-range restriction map of the interleukin-4 and interleukin-5 linkage group on chromosome 5. Genomics 6:94-99.

Chen CH, Zhang DH, LaPorte JM, Ray A (2000) Cyclic AMP activates p38 mitogen-activated protein kinase in Th2 cells: phosphorylation of GATA-3 and stimulation of Th2 cytokine gene expression. J Immunol 165:5597-5605.

Chen D, Rothenberg EV (1994) Interleukin 2 transcription factors as molecular targets of cAMP inhibition: delayed inhibition kinetics and combinatorial transcription roles. J Exp Med 179:931-942.

Chrivia JC, Kwok RP, Lamb N, Hagiwara M, Montminy MR, Goodman RH (1993) Phosphorylated CREB binds specifically to the nuclear protein CBP. Nature 365:855-859.

Chuvpilo S, Schomberg C, Gerwig R, Heinfling A, Reeves R, Grummt F, Serfling E (1993) Multiple closely-linked NFAT/octamer and HMG I(Y) binding sites are part of the interleukin-4 promoter. Nucleic Acids Res 21:5694-5704.

Clipstone NA, Crabtree GR (1992) Identification of calcineurin as a key signalling enzyme in T- lymphocyte activation. Nature 357:695-697.

Clutterbuck E, Shields JG, Gordon J, Smith SH, Boyd A, Callard RE, Campbell HD, Young IG, Sanderson CJ (1987) Recombinant human interleukin 5 is an eosinophil differentiation factor but has no activity in standard human B cell growth factor assays. Eur J Immunol 17:1743-1750.

Clutterbuck EJ, Hirst EM, Sanderson CJ (1989) Human interleukin-5 (IL-5) regulates the production of eosinophils in human bone marrow cultures: comparison and interaction with IL-1, IL-3, IL-6, and GMCSF. Blood 73:1504-1512.

Cockerill GW, Bert AG, Ryan GR, Gamble JR, Vadas MA, Cockerill PN (1995) Regulation of granulocyte-macrophage colony-stimulating factor and E- selectin expression in endothelial cells by cyclosporin A and the T- cell transcription factor NFAT. Blood 86:2689-2698.

Cockerill PN, Shannon MF, Bert AG, Ryan GR, Vadas MA (1993) The granulocyte-macrophage colony-stimulating factor/interleukin 3 locus is regulated by an inducible cyclosporin A-sensitive enhancer. Proc Natl Acad Sci U S A 90:2466-2470.

Coffman RL, Seymour BW, Hudak S, Jackson J, Rennick D (1989) Antibody to interleukin-5 inhibits helminth-induced eosinophilia in mice. Science 245:308-310.

Cogan E, Schandene L, Crusiaux A, Cochaux P, Velu T, Goldman M (1994) Brief report: clonal proliferation of type 2 helper T cells in a man with the hypereosinophilic syndrome. N Engl J Med 330:535-538.

Cohen DR, Curran T (1988) fra-1: a serum-inducible, cellular immediate-early gene that encodes a fos-related antigen. Mol Cell Biol 8:2063-2069.

Collins PD, Marleau S, Griffiths-Johnson DA, Jose PJ, Williams TJ (1995) Cooperation between interleukin-5 and the chemokine eotaxin to induce eosinophil accumulation in vivo. J Exp Med 182:1169-1174.

Conscience JF, Verrier B, Martin G (1986) Interleukin-3-dependent expression of the c-myc and c-fos proto- oncogenes in hemopoietic cell lines. Embo J 5:317-323.

Cornelis S, Plaetinck G, Devos R, Van der Heyden J, Tavernier J, Sanderson CJ, Guisez Y, Fiers W (1995) Detailed analysis of the IL-5-IL-5R alpha interaction: characterization of crucial residues on the ligand and the receptor. Embo J 14:3395-3402.

Crawley JB, Rawlinson L, Lali FV, Page TH, Saklatvala J, Foxwell BM (1997) T cell proliferation in response to interleukins 2 and 7 requires p38MAP kinase activation. J Biol Chem 272:15023-15027.

Daly CM, Mayrhofer G, Dent LA (1999) Trapping and immobilization of Nippostrongylus brasiliensis larvae at the site of inoculation in primary infections of interleukin-5 transgenic mice. Infect Immun 67:5315-5323.

Danna KJ, Sompayrac LM (1982) Efficient infection of monkey cells with SV40 DNA. II. Use of low- molecular-weight DEAE-dextran for large-scale experiments. J Virol Methods 5:335-341.

Daum G, Eisenmann-Tappe I, Fries HW, Troppmair J, Rapp UR (1994) The ins and outs of Raf kinases. Trends Biochem Sci 19:474-480.

Dent LA, Strath M, Mellor AL, Sanderson CJ (1990) Eosinophilia in transgenic mice expressing interleukin 5. J Exp Med 172:1425-1431.

Dent LA, Daly CM, Mayrhofer G, Zimmerman T, Hallett A, Bignold LP, Creaney J, Parsons JC (1999) Interleukin-5 transgenic mice show enhanced resistance to primary infections with Nippostrongylus brasiliensis but not primary infections with Toxocara canis. Infect Immun 67:989-993.

Derigs HG, Burgess GS, Klingberg D, Nahreini TS, Mochizuki DY, Williams DE, Boswell HS (1990) Role for cyclic AMP in the postreceptor control of cytokine-stimulated stromal cell growth factor production. Leukemia 4:471-479.

Derijard B, Raingeaud J, Barrett T, Wu IH, Han J, Ulevitch RJ, Davis RJ (1995) Independent human MAP-kinase signal transduction pathways defined by MEK and MKK isoforms. Science 267:682-685.

Dickens M, Rogers JS, Cavanagh J, Raitano A, Xia Z, Halpern JR, Greenberg ME, Sawyers CL, Davis RJ (1997) A cytoplasmic inhibitor of the JNK signal transduction pathway. Science 277:693-696.

Du W, Thanos D, Maniatis T (1993) Mechanisms of transcriptional synergism between distinct virus- inducible enhancer elements. Cell 74:887-898.

Dubucquoi S, Desreumaux P, Janin A, Klein O, Goldman M, Tavernier J, Capron A, Capron M (1994) Interleukin 5 synthesis by eosinophils: association with granules and immunoglobulin-dependent secretion. J Exp Med 179:703-708.

Duronio V, Clark-Lewis I, Federsppiel B, Wieler JS, Schrader JW (1992) Tyrosine phosphorylation of receptor beta subunits and common substrates in response to interleukin-3 and granulocyte-macrophage colony-stimulating factor. J Biol Chem 267:21856-21863.

Egan RW, Athwahl D, Chou CC, Emtage S, Jehn CH, Kung TT, Mauser PJ, Murgolo NJ, Bodmer MW (1995) Inhibition of pulmonary eosinophilia and hyperreactivity by antibodies to interleukin-5. Int Arch Allergy Immunol 107:321-322.

Egan SE, Giddings BW, Brooks MW, Buday L, Sizeland AM, Weinberg RA (1993) Association of Sos Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. Nature 363:45-51.

Egerton M, Fitzpatrick DR, Kelso A (1998) Activation of the extracellular signal-regulated kinase pathway is differentially required for TCR-stimulated production of six cytokines in primary T lymphocytes. Int Immunol 10:223-229.

Emson CL, Bell SE, Jones A, Wisden W, McKenzie AN (1998) Interleukin (IL)-4-independent induction of immunoglobulin (Ig)E, and perturbation of T cell development in transgenic mice expressing IL-13. J Exp Med 188:399-404.

Enslen H, Raingeaud J, Davis RJ (1998) Selective activation of p38 mitogen-activated protein (MAP) kinase isoforms by the MAP kinase kinases MKK3 and MKK6. J Biol Chem 273:1741-1748.

Ernst P, Smale ST (1995) Combinatorial regulation of transcription. I: General aspects of transcriptional control. Immunity 2:311-319.

Evans T, Felsenfeld G (1991) trans-Activation of a globin promoter in nonerythroid cells. Mol Cell Biol 11:843-853.

Falvo JV, Thanos D, Maniatis T (1995) Reversal of intrinsic DNA bends in the IFN beta gene enhancer by transcription factors and the architectural protein HMG I(Y). Cell 83:1101-1111.

Fanger GR, Gerwins P, Widmann C, Jarpe MB, Johnson GL (1997) MEKKs, GCKs, MLKs, PAKs, TAKs, and tpls: upstream regulators of the c- Jun amino-terminal kinases? Curr Opin Genet Dev 7:67-74.

Felgner PL, Gadek TR, Holm M, Roman R, Chan HW, Wenz M, Northrop JP, Ringold GM, Danielsen M (1987) Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc Natl Acad Sci U S A 84:7413-7417.

Fitzpatrick DR, Shirley KM, Kelso A (1999) Cutting edge: stable epigenetic inheritance of regional IFN-gamma promoter demethylation in CD44highCD8+ T lymphocytes. J Immunol 162:5053-5057.

Folkard SG, Hogarth PJ, Taylor MJ, Bianco AE (1996) Eosinophils are the major effector cells of immunity to microfilariae in a mouse model of onchocerciasis. Parasitology 112:323-329.

Foster PF, Sankary HN, Hart M, Ashmann M, Williams JW (1989) Blood and graft eosinophilia as predictors of rejection in human liver transplantation. Transplantation 47:72-74.

Foster PS, Hogan SP, Ramsay AJ, Matthaei KI, Young IG (1996) Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model. J Exp Med 183:195-201.

Foulkes NS, Sassone-Corsi P (1996) Transcription factors coupled to the cAMP-signalling pathway. Biochim Biophys Acta 1288:F101-121.

Fraser JK, Tran S, Nimer SD, Gasson JC (1994) Characterization of nuclear factors that bind to a critical positive regulatory element of the human granulocyte-macrophage colony- stimulating factor promoter. Blood 84:2523-2530.

Fried M, Crothers DM (1981) Equilibria and kinetics of lac repressor-operator interactions by polyacrylamide gel electrophoresis. Nucleic Acids Res 9:6505-6525.

Fujisawa T, Abu-Ghazaleh R, Kita H, Sanderson CJ, Gleich GJ (1990) Regulatory effect of cytokines on eosinophil degranulation. J Immunol 144:642-646.

Galli SJ (1997) Complexity and redundancy in the pathogenesis of asthma: reassessing the roles of mast cells and T cells. J Exp Med 186:343-347.

Garner MM, Revzin A (1981) A gel electrophoresis method for quantifying the binding of proteins to specific DNA regions: application to components of the Escherichia coli lactose operon regulatory system. Nucleic Acids Res 9:3047-3060.

Gegonne A, Leprince D, Duterque-Coquillaud M, Vandenbunder B, Flourens A, Ghysdael J, Debuire B, Stehelin D (1987) Multiple domains for the chicken cellular sequences homologous to the v- ets oncogene of the E26 retrovirus. Mol Cell Biol 7:806-812.

Gorman CM, Moffat LF, Howard BH (1982) Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol Cell Biol 2:1044-1051.

Gorman DM, Itoh N, Jenkins NA, Gilbert DJ, Copeland NG, Miyajima A (1992) Chromosomal localization and organization of the murine genes encoding the beta subunits (AIC2A and AIC2B) of the interleukin 3, granulocyte/macrophage colony-stimulating factor, and interleukin 5 receptors. J Biol Chem 267:15842-15848.

Gottschalk LR, Giannola DM, Emerson SG (1993) Molecular regulation of the human IL-3 gene: inducible T cell- restricted expression requires intact AP-1 and Elf-1 nuclear protein binding sites. J Exp Med 178:1681-1692.

Griffiths MR, Black EJ, Culbert AA, Dickens M, Shaw PE, Gillespie DA, Tavare JM (1998) Insulin-stimulated expression of c-fos, fra1 and c-jun accompanies the activation of the activator protein-1 (AP-1) transcriptional complex. Biochem J 335:19-26.

Grimaldi JC, Meeker TC (1989) The t(5;14) chromosomal translocation in a case of acute lymphocytic leukemia joins the interleukin-3 gene to the immunoglobulin heavy chain gene. Blood 73:2081-2085.

Griswold DE, Hillegass LM, Meunier PC, DiMartino MJ, Hanna N (1988) Effect of inhibitors of eicosanoid metabolism in murine collagen- induced arthritis. Arthritis Rheum 31:1406-1412.

Gruart-Gouilleux V, Engels P, Sullivan M (1995) Characterization of the human interleukin-5 gene promoter: involvement of octamer binding sites in the gene promoter activity. Eur J Immunol 25:1431-1435.

Grunig G, Warnock M, Wakil AE, Venkayya R, Brombacher F, Rennick DM, Sheppard D, Mohrs M, Donaldson DD, Locksley RM, Corry DB (1998) Requirement for IL-13 independently of IL-4 in experimental asthma. Science 282:2261-2263.

Gupta S, Barrett T, Whitmarsh AJ, Cavanagh J, Sluss HK, Derijard B, Davis RJ (1996) Selective interaction of JNK protein kinase isoforms with transcription factors. Embo J 15:2760-2770.

Hagan P, Moore PJ, Adjukiewicz AB, Greenwood BM, Wilkins HA (1985) In-vitro antibody-dependent killing of schistosomula of Schistosoma haematobium by human eosinophils. Parasite Immunol 7:617-624.

Hai T, Curran T (1991) Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity. Proc Natl Acad Sci U S A 88:3720-3724.

Hale KK, Trollinger D, Rihanek M, Manthey CL (1999) Differential expression and activation of p38 mitogen-activated protein kinase alpha, beta, gamma, and delta in inflammatory cell lineages. J Immunol 162:4246-4252.

Hamid Q, Azzawi M, Ying S, Moqbel R, Wardlaw AJ, Corrigan CJ, Bradley B, Durham SR, Collins JV, Jeffery PK, et al. (1991) Expression of mRNA for interleukin-5 in mucosal bronchial biopsies from asthma. J Clin Invest 87:1541-1546.

Han J, Lee JD, Bibbs L, Ulevitch RJ (1994) A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. Science 265:808-811.

Han J, Lee JD, Jiang Y, Li Z, Feng L, Ulevitch RJ (1996) Characterization of the structure and function of a novel MAP kinase kinase (MKK6). J Biol Chem 271:2886-2891.

Hendriks RW, Nawijn MC, Engel JD, van Doorninck H, Grosveld F, Karis A (1999) Expression of the transcription factor GATA-3 is required for the development of the earliest T cell progenitors and correlates with stages of cellular proliferation in the thymus. Eur J Immunol 29:1912-1918.

Himes SR, Coles LS, Reeves R, Shannon MF (1996) High mobility group protein I(Y) is required for function and for c-Rel binding to CD28 response elements within the GM-CSF and IL-2 promoters. Immunity 5:479-489.

Himes SR, Reeves R, Attema J, Nissen M, Li Y, Shannon MF (2000) The role of high-mobility group I(Y) proteins in expression of IL-2 and T cell proliferation. J Immunol 164:3157-3168.

Hipskind RA, Rao VN, Mueller CG, Reddy ES, Nordheim A (1991) Ets-related protein Elk-1 is homologous to the c-fos regulatory factor p62TCF. Nature 354:531-534.

Hitoshi Y, Yamaguchi N, Mita S, Sonoda E, Takaki S, Tominaga A, Takatsu K (1990) Distribution of IL-5 receptor-positive B cells. Expression of IL-5 receptor on Ly-1(CD5)+ B cells. J Immunol 144:4218-4225.

Ho IC, Vorhees P, Marin N, Oakley BK, Tsai SF, Orkin SH, Leiden JM (1991) Human GATA-3: a lineage-restricted transcription factor that regulates the expression of the T cell receptor alpha gene. Embo J 10:1187-1192.

Hobbs S, Jitrapakdee S, Wallace JC (1998) Development of a bicistronic vector driven by the human polypeptide chain elongation factor 1alpha promoter for creation of stable mammalian cell lines that express very high levels of recombinant proteins. Biochem Biophys Res Commun 252:368-372.

Hodge MR, Chun HJ, Rengarajan J, Alt A, Lieberson R, Glimcher LH (1996) NF-AT-Driven interleukin-4 transcription potentiated by NIP45. Science 274:1903-1905.

Hoey T, Sun YL, Williamson K, Xu X (1995) Isolation of two new members of the NF-AT gene family and functional characterization of the NF-AT proteins. Immunity 2:461-472.

Hoffmeyer A, Avots A, Flory E, Weber CK, Serfling E, Rapp UR (1998) The GABP-responsive element of the interleukin-2 enhancer is regulated by JNK/SAPK-activating pathways in T lymphocytes. J Biol Chem 273:10112-10119.

Hoffmeyer A, Grosse-Wilde A, Flory E, Neufeld B, Kunz M, Rapp UR, Ludwig S (1999) Different mitogen-activated protein kinase signaling pathways cooperate to regulate tumor necrosis factor alpha gene expression in T lymphocytes. J Biol Chem 274:4319-4327.

Hoidal JR (1990) The eosinophil and acute lung injury. Am Rev Respir Dis 142:1245-1246.

Huston MM, Moore JP, Mettes HJ, Tavana G, Huston DP (1996) Human B cells express IL-5 receptor messenger ribonucleic acid and respond to IL-5 with enhanced IgM production after mitogenic stimulation with Moraxella catarrhalis. J Immunol 156:1392-1401.

Huth JR, Bewley CA, Nissen MS, Evans JN, Reeves R, Gronenborn AM, Clore GM (1997) The solution structure of an HMG-I(Y)-DNA complex defines a new architectural minor groove binding motif. Nat Struct Biol 4:657-665.

Ihle JN (1995) The Janus protein tyrosine kinase family and its role in cytokine signaling. Adv Immunol 60:1-35.

Ip YT, Davis RJ (1998) Signal transduction by the c-Jun N-terminal kinase (JNK)--from inflammation to development. Curr Opin Cell Biol 10:205-219.

Janknecht R, Hunter T (1996) Transcription. A growing coactivator network. Nature 383:22-23.

Jenkins MK, DeSilva DR, Johnson JG, Norton SD (1993) Costimulating factors and signals relevant for antigen presenting cell function. Adv Exp Med Biol 329:87-92.

Jiang Y, Gram H, Zhao M, New L, Gu J, Feng L, Di Padova F, Ulevitch RJ, Han J (1997) Characterization of the structure and function of the fourth member of p38 group mitogen-activated protein kinases, p38delta. J Biol Chem 272:30122-30128.

Johanson K, Appelbaum E, Doyle M, Hensley P, Zhao B, Abdel-Meguid SS, Young P, Cook R, Carr S, Matico R, et al. (1995) Binding interactions of human interleukin 5 with its receptor alpha subunit. Large scale production, structural, and functional studies of Drosophila-expressed recombinant proteins. J Biol Chem 270:9459-9471.

John S, Robbins CM, Leonard WJ (1996) An IL-2 response element in the human IL-2 receptor alpha chain promoter is a composite element that binds Stat5, Elf-1, HMG-I(Y) and a GATA family protein. Embo J 15:5627-5635.

John S, Reeves RB, Lin JX, Child R, Leiden JM, Thompson CB, Leonard WJ (1995) Regulation of cell-type-specific interleukin-2 receptor alpha-chain gene expression: potential role of physical interactions between Elf-1, HMG-I(Y), and NF-kappa B family proteins. Mol Cell Biol 15:1786-1796.

Kadonaga JT (1998) Eukaryotic transcription: an interlaced network of transcription factors and chromatin-modifying machines. Cell 92:307-313.

Kaplan MH, Sun YL, Hoey T, Grusby MJ (1996) Impaired IL-12 responses and enhanced development of Th2 cells in Stat4- deficient mice. Nature 382:174-177.

Karim FD, Urness LD, Thummel CS, Klemsz MJ, McKercher SR, Celada A, Van Beveren C, Maki RA, Gunther CV, Nye JA, et al. (1990) The ETS-domain: a new DNA-binding motif that recognizes a purine-rich core DNA sequence. Genes Dev 4:1451-1453.

Karin M (1995) The regulation of AP-1 activity by mitogen-activated protein kinases. J Biol Chem 270:16483-16486.

Karlen S, D'Ercole M, Sanderson CJ (1996) Two pathways can activate the interleukin-5 gene and induce binding to the conserved lymphokine element 0. Blood 88:211-221.

Kay AB, Ying S, Durham SR (1995) Phenotype of cells positive for interleukin-4 and interleukin-5 mRNA in allergic tissue reactions. Int Arch Allergy Immunol 107:208-210.

Kaye J, Porcelli S, Tite J, Jones B, Janeway CA, Jr. (1983) Both a monoclonal antibody and antisera specific for determinants unique to individual cloned helper T cell lines can substitute for antigen and antigen-presenting cells in the activation of T cells. J Exp Med 158:836-856.

Kees UR, Peroni SE, Ranford PR, Ford J (1994) A new human cell line for the molecular dissection of the requirement for interleukin-1 in the production of interleukin-2 by immature T cells. J Immunol Methods 168:1-8.

Kim J, Reeves R, Rothman P, Boothby M (1995) The non-histone chromosomal protein HMG-I(Y) contributes to repression of the immunoglobulin heavy chain germ-line epsilon RNA promoter. Eur J Immunol 25:798-808.

Kim JI, Ho IC, Grusby MJ, Glimcher LH (1999) The transcription factor c-Maf controls the production of interleukin-4 but not other Th2 cytokines. Immunity 10:745-751.

Kinashi T, Harada N, Severinson E, Tanabe T, Sideras P, Konishi M, Azuma C, Tominaga A, Bergstedt-Lindqvist S, Takahashi M, et al. (1986) Cloning of

complementary DNA encoding T-cell replacing factor and identity with B-cell growth factor II. Nature 324:70-73.

Klein-Hessling S, Schneider G, Heinfling A, Chuvpilo S, Serfling E (1996) HMG I(Y) interferes with the DNA binding of NF-AT factors and the induction of the interleukin 4 promoter in T cells. Proc Natl Acad Sci U S A 93:15311-15316.

Klemsz MJ, McKercher SR, Celada A, Van Beveren C, Maki RA (1990) The macrophage and B cell-specific transcription factor PU.1 is related to the ets oncogene. Cell 61:113-124.

Klenchin VA, Sukharev SI, Serov SM, Chernomordik LV, Chizmadzhev Yu A (1991) Electrically induced DNA uptake by cells is a fast process involving DNA electrophoresis. Biophys J 60:804-811.

Ko LJ, Engel JD (1993) DNA-binding specificities of the GATA transcription factor family. Mol Cell Biol 13:4011-4022.

Kondo T, Marchevsky AM, Jordan SC, Koerner SK, Matloff JM, Waters PF (1991) Vascular rejection and graft eosinophilia in rat lung allografts. J Surg Res 51:310-315.

Kopf M, Le Gros G, Bachmann M, Lamers MC, Bluethmann H, Kohler G (1993) Disruption of the murine IL-4 gene blocks Th2 cytokine responses. Nature 362:245-248.

Kopf M, Brombacher F, Hodgkin PD, Ramsay AJ, Milbourne EA, Dai WJ, Ovington KS, Behm CA, Kohler G, Young IG, Matthaei KI (1996) IL-5-deficient mice have a developmental defect in CD5+ B-1 cells and lack eosinophilia but have normal antibody and cytotoxic T cell responses. Immunity 4:15-24.

Korenaga M, Hitoshi Y, Takatsu K, Tada I (1994) Regulatory effect of anti-interleukin-5 monoclonal antibody on intestinal worm burden in a primary infection with strongyloides venezuelensis in mice. Int J Parasitol 24:951-957.

Kormendi F, Amend WJ, Jr. (1988) The importance of eosinophil cells in kidney allograft rejection. Transplantation 45:537-539.

Kouro T, Kikuchi Y, Kanazawa H, Hirokawa K, Harada N, Shiiba M, Wakao H, Takaki S, Takatsu K (1996) Critical proline residues of the cytoplasmic domain of the IL-5 receptor alpha chain and its function in IL-5-mediated activation of JAK kinase and STAT5. Int Immunol 8:237-245.

Kuhn R, Rajewsky K, Muller W (1991) Generation and analysis of interleukin-4 deficient mice. Science 254:707-710.

Kung TT, Stelts DM, Zurcher JA, Jones H, Umland SP, Egan RW, Kreutner W, Chapman RW (1995) Interferon-gamma and antibodies to interleukin-5 and interleukin-4 inhibit the pulmonary eosinophilia in allergic mice. Inflamm Res 44 Suppl 2:S185-186.

Kwok RP, Lundblad JR, Chrivia JC, Richards JP, Bachinger HP, Brennan RG, Roberts SG, Green MR, Goodman RH (1994) Nuclear protein CBP is a coactivator for the transcription factor CREB. Nature 370:223-226.

Kyriakis JM, Banerjee P, Nikolakaki E, Dai T, Rubie EA, Ahmad MF, Avruch J, Woodgett JR (1994) The stress-activated protein kinase subfamily of c-Jun kinases. Nature 369:156-160.

Lai JH, Tan TH (1994) CD28 signaling causes a sustained down-regulation of I kappa B alpha which can be prevented by the immunosuppressant rapamycin. J Biol Chem 269:30077-30080.

LaMarco K, Thompson CC, Byers BP, Walton EM, McKnight SL (1991) Identification of Ets- and notch-related subunits in GA binding protein. Science 253:789-792.

Landschulz WH, Johnson PF, McKnight SL (1988) The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. Science 240:1759-1764.

Larner AC, David M, Feldman GM, Igarashi K, Hackett RH, Webb DS, Sweitzer SM, Petricoin EF, 3rd, Finbloom DS (1993) Tyrosine phosphorylation of DNA binding proteins by multiple cytokines. Science 261:1730-1733.

Laverriere AC, MacNeill C, Mueller C, Poelmann RE, Burch JB, Evans T (1994) GATA-4/5/6, a subfamily of three transcription factors transcribed in developing heart and gut. J Biol Chem 269:23177-23184.

Le Beau MM, Lemons RS, Espinosa R, 3rd, Larson RA, Arai N, Rowley JD (1989) Interleukin-4 and interleukin-5 map to human chromosome 5 in a region encoding growth factors and receptors and are deleted in myeloid leukemias with a del(5q). Blood 73:647-650.

Le Moine A, Flamand V, Demoor FX, Noel JC, Surquin M, Kiss R, Nahori MA, Pretolani M, Goldman M, Abramowicz D (1999a) Critical roles for IL-4, IL-5, and eosinophils in chronic skin allograft rejection. J Clin Invest 103:1659-1667.

Le Moine A, Surquin M, Demoor FX, Noel JC, Nahori MA, Pretolani M, Flamand V, Braun MY, Goldman M, Abramowicz D (1999b) IL-5 mediates eosinophilic rejection of MHC class II-disparate skin allografts in mice. J Immunol 163:3778-3784.

Lecine P, Algarte M, Rameil P, Beadling C, Bucher P, Nabholz M, Imbert J (1996) Elf-1 and Stat5 bind to a critical element in a new enhancer of the human interleukin-2 receptor alpha gene. Mol Cell Biol 16:6829-6840.

Lee HJ, O'Garra A, Arai K, Arai N (1998) Characterization of cis-regulatory elements and nuclear factors conferring Th2-specific expression of the IL-5 gene: a role for a GATA- binding protein. J Immunol 160:2343-2352.

Lee HJ, Masuda ES, Arai N, Arai K, Yokota T (1995) Definition of cis-regulatory elements of the mouse interleukin-5 gene promoter. Involvement of nuclear factor of activated T cell-related factors in interleukin-5 expression. J Biol Chem 270:17541-17550.

Lee HJ, Matsuda I, Naito Y, Yokota T, Arai N, Arai K (1994a) Signals and nuclear factors that regulate the expression of interleukin- 4 and interleukin-5 genes in helper T cells. J Allergy Clin Immunol 94:594-604.

Lee HJ, Koyano-Nakagawa N, Naito Y, Nishida J, Arai N, Arai K, Yokota T (1993) cAMP activates the IL-5 promoter synergistically with phorbol ester through the signaling pathway involving protein kinase A in mouse thymoma line EL-4. J Immunol 151:6135-6142.

Lee JC, Laydon JT, McDonnell PC, Gallagher TF, Kumar S, Green D, McNulty D, Blumenthal MJ, Heys JR, Landvatter SW, et al. (1994b) A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. Nature 372:739-746.

Lee JS, Campbell HD, Kozak CA, Young IG (1989) The IL-4 and IL-5 genes are closely linked and are part of a cytokine gene cluster on mouse chromosome 11. Somat Cell Mol Genet 15:143-152.

Leiden JM, Wang CY, Petryniak B, Markovitz DM, Nabel GJ, Thompson CB (1992) A novel Ets-related transcription factor, Elf-1, binds to human immunodeficiency virus

type 2 regulatory elements that are required for inducible trans activation in T cells. J Virol 66:5890-5897.

Li-Weber M, Salgame P, Hu C, Davydov IV, Laur O, Klevenz S, Krammer PH (1998) Th2-specific protein/DNA interactions at the proximal nuclear factor-AT site contribute to the functional activity of the human IL-4 promoter. J Immunol 161:1380-1389.

Liu P, Tarle SA, Hajra A, Claxton DF, Marlton P, Freedman M, Siciliano MJ, Collins FS (1993) Fusion between transcription factor CBF beta/PEBP2 beta and a myosin heavy chain in acute myeloid leukemia. Science 261:1041-1044.

Loots GG, Locksley RM, Blankespoor CM, Wang ZE, Miller W, Rubin EM, Frazer KA (2000) Identification of a coordinate regulator of interleukins 4, 13, and 5 by cross-species sequence comparisons. Science 288:136-140.

Lopez AF, Elliott MJ, Woodcock J, Vadas MA (1992) GM-CSF, IL-3 and IL-5: cross-competition on human haemopoietic cells. Immunol Today 13:495-500.

Lowe D, Jorizzo J, Hutt MS (1981) Tumour-associated eosinophilia: a review. J Clin Pathol 34:1343-1348.

Macatonia SE, Hsieh CS, Murphy KM, O'Garra A (1993) Dendritic cells and macrophages are required for Th1 development of CD4+ T cells from alpha beta TCR transgenic mice: IL-12 substitution for macrophages to stimulate IFN-gamma production is IFN-gamma- dependent. Int Immunol 5:1119-1128.

Macleod K, Plumb M (1991) Derepression of mouse beta-major-globin gene transcription during erythroid differentiation. Mol Cell Biol 11:4324-4332.

Magram J, Connaughton SE, Warrier RR, Carvajal DM, Wu CY, Ferrante J, Stewart C, Sarmiento U, Faherty DA, Gately MK (1996) IL-12-deficient mice are defective in IFN gamma production and type 1 cytokine responses. Immunity 4:471-481.

Mahanty S, Nutman TB (1993) The biology of interleukin-5 and its receptor. Cancer Invest 11:624-634.

Mandler R, Chu CC, Paul WE, Max EE, Snapper CM (1993) Interleukin 5 induces S mu-S gamma 1 DNA rearrangement in B cells activated with dextran-anti-IgD antibodies and interleukin 4: a three component model for Ig class switching. J Exp Med 178:1577-1586.

Marrugo J, Marsh DG, Ghosh B. (1996) The conserved lymphokine element-0 in the IL5 promoter binds to a high mobility group-1 protein. Mol Immunol 33(14):1119-25

Manetti R, Parronchi P, Giudizi MG, Piccinni MP, Maggi E, Trinchieri G, Romagnani S (1993) Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4-producing Th cells. J Exp Med 177:1199-1204.

Martinez OM, Krams SM, Sterneck M, Villanueva JC, Falco DA, Ferrell LD, Lake J, Roberts JP, Ascher NL (1992) Intragraft cytokine profile during human liver allograft rejection. Transplantation 53:449-456.

Masuda ES, Tokumitsu H, Tsuboi A, Shlomai J, Hung P, Arai K, Arai N (1993) The granulocyte-macrophage colony-stimulating factor promoter cis- acting element CLE0 mediates induction signals in T cells and is recognized by factors related to AP1 and NFAT. Mol Cell Biol 13:7399-7407.

Masuda ES, Naito Y, Tokumitsu H, Campbell D, Saito F, Hannum C, Arai K, Arai N (1995) NFATx, a novel member of the nuclear factor of activated T cells family that is expressed predominantly in the thymus. Mol Cell Biol 15:2697-2706.

Mauser PJ, Pitman AM, Fernandez X, Foran SK, Adams GK, 3rd, Kreutner W, Egan RW, Chapman RW (1995) Effects of an antibody to interleukin-5 in a monkey model of asthma. Am J Respir Crit Care Med 152:467-472.

McKenzie AN, Barry SC, Strath M, Sanderson CJ (1991) Structure-function analysis of interleukin-5 utilizing mouse/human chimeric molecules. Embo J 10:1193-1199.

McKenzie AN, Li X, Largaespada DA, Sato A, Kaneda A, Zurawski SM, Doyle EL, Milatovich A, Francke U, Copeland NG, et al. (1993) Structural comparison and chromosomal localization of the human and mouse IL-13 genes. J Immunol 150:5436-5444.

McKenzie DT, Filutowicz HI, Swain SL, Dutton RW (1987) Purification and partial sequence analysis of murine B cell growth factor II (interleukin 5). J Immunol 139:2661-2668.

McKenzie GJ, Emson CL, Bell SE, Anderson S, Fallon P, Zurawski G, Murray R, Grencis R, McKenzie AN (1998) Impaired development of Th2 cells in IL-13-deficient mice. Immunity 9:423-432.

Meraz MA, White JM, Sheehan KC, Bach EA, Rodig SJ, Dighe AS, Kaplan DH, Riley JK, Greenlund AC, Campbell D, Carver-Moore K, DuBois RN, Clark R, Aguet M,

Schreiber RD (1996) Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. Cell 84:431-442.

Merika M, Orkin SH (1993) DNA-binding specificity of GATA family transcription factors. Mol Cell Biol 13:3999-4010.

Milburn MV, Hassell AM, Lambert MH, Jordan SR, Proudfoot AE, Graber P, Wells TN (1993) A novel dimer configuration revealed by the crystal structure at 2.4 A resolution of human interleukin-5. Nature 363:172-176.

Minamitake Y, Kodama S, Katayama T, Adachi H, Tanaka S, Tsujimoto M (1990) Structure of recombinant human interleukin 5 produced by Chinese hamster ovary cells. J Biochem (Tokyo) 107:292-297.

Minty A, Chalon P, Derocq JM, Dumont X, Guillemot JC, Kaghad M, Labit C, Leplatois P, Liauzun P, Miloux B, et al. (1993) Interleukin-13 is a new human lymphokine regulating inflammatory and immune responses. Nature 362:248-250.

Mita S, Takaki S, Tominaga A, Takatsu K (1993) Comparative analysis of the kinetics of binding and internalization of IL-5 in murine IL-5 receptors of high and low affinity. J Immunol 151:6924-6932.

Miyatake S, Shlomai J, Arai K, Arai N (1991) Characterization of the mouse granulocyte-macrophage colony-stimulating factor (GM-CSF) gene promoter: nuclear factors that interact with an element shared by three lymphokine genes--those for GM-CSF, interleukin- 4 (IL-4), and IL-5. Mol Cell Biol 11:5894-5901.

Mizushima S, Nagata S (1990) pEF-BOS, a powerful mammalian expression vector. Nucleic Acids Res 18:5322.

Mizuta TR, Tanabe T, Nakakubo H, Noma T, Honjo T (1988) Molecular cloning and structure of the mouse interleukin-5 gene. Growth Factors 1:51-57.

Mordvinov VA, Peroni SE, De Boer ML, Kees UR, Sanderson CJ (1999) A human T-cell line with inducible production of interleukins 5 and 4. A model for studies of gene expression. J Immunol Methods 228:163-168.

Morgan JG, Dolganov GM, Robbins SE, Hinton LM, Lovett M (1992) The selective isolation of novel cDNAs encoded by the regions surrounding the human interleukin 4 and 5 genes. Nucleic Acids Res 20:5173-5179.

Mori A, Kaminuma O, Miyazawa K, Ogawa K, Okudaira H, Akiyama K (1999) p38 mitogen-activated protein kinase regulates human T cell IL-5 synthesis. J Immunol 163:4763-4771.

Morla AO, Schreurs J, Miyajima A, Wang JY (1988) Hematopoietic growth factors activate the tyrosine phosphorylation of distinct sets of proteins in interleukin-3-dependent murine cell lines. Mol Cell Biol 8:2214-2218.

Mosmann TR, Coffman RL (1989) TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol 7:145-173.

Mould AW, Matthaei KI, Young IG, Foster PS (1997) Relationship between interleukin-5 and eotaxin in regulating blood and tissue eosinophilia in mice. J Clin Invest 99:1064-1071.

Mui AL, Wakao H, O'Farrell AM, Harada N, Miyajima A (1995) Interleukin-3, granulocyte-macrophage colony stimulating factor and interleukin-5 transduce signals through two STAT5 homologs. Embo J 14:1166-1175.

Munoz E, Beutner U, Zubiaga A, Huber BT (1990) IL-1 activates two separate signal transduction pathways in T helper type II cells. J Immunol 144:964-969.

Murata Y, Yamaguchi N, Hitoshi Y, Tominaga A, Takatsu K (1990) Interleukin 5 and interleukin 3 induce serine and tyrosine phosphorylations of several cellular proteins in an interleukin 5- dependent cell line. Biochem Biophys Res Commun 173:1102-1108.

Murata Y, Takaki S, Migita M, Kikuchi Y, Tominaga A, Takatsu K (1992) Molecular cloning and expression of the human interleukin 5 receptor. J Exp Med 175:341-351.

Murphy E, Shibuya K, Hosken N, Openshaw P, Maino V, Davis K, Murphy K, O'Garra A (1996) Reversibility of T helper 1 and 2 populations is lost after long-term stimulation. J Exp Med 183:901-913.

Nagai H, Yamaguchi S, Inagaki N, Tsuruoka N, Hitoshi Y, Takatsu K (1993) Effect of anti-IL-5 monoclonal antibody on allergic bronchial eosinophilia and airway hyperresponsiveness in mice. Life Sci 53:L243-247.

Naora H (1993) Regulation of lymphokine gene expression in activated murine T-lymphocytes. PhD Thesis Australian National University.

Naora H, Young IG (1994b) Mechanisms regulating the mRNA levels of interleukin-5 and two other coordinately expressed lymphokines in the murine T lymphoma EL4.23. Blood 83:3620-3628.

Naora H, Young IG (1995) Comparison of the mechanisms regulating IL-5, IL-4, and three other lymphokine genes in the Th2 clone D10.G4.1. Exp Hematol 23:597-602.

Naora H, van Leeuwen BH, Bourke PF, Young IG (1994a) Functional role and signal-induced modulation of proteins recognizing the conserved TCATTT-containing promoter elements in the murine IL-5 and GM-CSF genes in T lymphocytes. J Immunol 153:3466-3475.

Nimer S, Fraser J, Richards J, Lynch M, Gasson J (1990) The repeated sequence CATT(A/T) is required for granulocyte-macrophage colony-stimulating factor promoter activity. Mol Cell Biol 10:6084-6088.

Nimer SD, Morita EA, Martis MJ, Wachsman W, Gasson JC (1988) Characterization of the human granulocyte-macrophage colony-stimulating factor promoter region by genetic analysis: correlation with DNase I footprinting. Mol Cell Biol 8:1979-1984.

Nishina H, Sato H, Suzuki T, Sato M, Iba H (1990) Isolation and characterization of fra-2, an additional member of the fos gene family. Proc Natl Acad Sci U S A 87:3619-3623.

Nishizuka Y (1986) Perspectives on the role of protein kinase C in stimulus-response coupling. J Natl Cancer Inst 76:363-370.

Nissen MS, Reeves R (1995) Changes in superhelicity are introduced into closed circular DNA by binding of high mobility group protein I/Y. J Biol Chem 270:4355-4360.

Noben-Trauth N, Shultz LD, Brombacher F, Urban JF, Jr., Gu H, Paul WE (1997) An interleukin 4 (IL-4)-independent pathway for CD4+ T cell IL-4 production is revealed in IL-4 receptor-deficient mice. Proc Natl Acad Sci U S A 94:10838-10843.

Nordeen SK (1988) Luciferase reporter gene vectors for analysis of promoters and enhancers. Biotechniques 6:454-458.

Novak TJ, Rothenberg EV (1990) cAMP inhibits induction of interleukin 2 but not of interleukin 4 in T cells. Proc Natl Acad Sci U S A 87:9353-9357.

Nunn MF, Seeburg PH, Moscovici C, Duesberg PH (1983) Tripartite structure of the avian erythroblastosis virus E26 transforming gene. Nature 306:391-395.

Ogata N, Kikuchi Y, Kouro T, Tomonaga M, Takatsu K (1997) The activation of the JAK2/STAT5 pathway is commonly involved in signaling through the human IL-5 receptor. Int Arch Allergy Immunol 114 Suppl 1:24-27.

Ogata N, Kouro T, Yamada A, Koike M, Hanai N, Ishikawa T, Takatsu K (1998) JAK2 and JAK1 constitutively associate with an interleukin-5 (IL-5) receptor alpha and betac subunit, respectively, and are activated upon IL-5 stimulation. Blood 91:2264-2271.

Ohnishi T, Kita H, Weiler D, Sur S, Sedgwick JB, Calhoun WJ, Busse WW, Abrams JS, Gleich GJ (1993) IL-5 is the predominant eosinophil-active cytokine in the antigen-induced pulmonary late-phase reaction. Am Rev Respir Dis 147:901-907.

Okamura H, Tsutsi H, Komatsu T, Yutsudo M, Hakura A, Tanimoto T, Torigoe K, Okura T, Nukada Y, Hattori K, et al. (1995) Cloning of a new cytokine that induces IFN-gamma production by T cells. Nature 378:88-91.

Olive M, Krylov D, Echlin DR, Gardner K, Taparowsky E, Vinson C (1997) A dominant negative to activation protein-1 (AP1) that abolishes DNA binding and inhibits oncogenesis. J Biol Chem 272:18586-18594.

Oosterwegel M, Timmerman J, Leiden J, Clevers H (1992) Expression of GATA-3 during lymphocyte differentiation and mouse embryogenesis. Dev Immunol 3:1-11.

Ouyang W, Ranganath SH, Weindel K, Bhattacharya D, Murphy TL, Sha WC, Murphy KM (1998) Inhibition of Th1 development mediated by GATA-3 through an IL-4-independent mechanism. Immunity 9:745-755.

Ouyang W, Lohning M, Gao Z, Assenmacher M, Ranganath S, Radbruch A, Murphy KM (2000) Stat6-independent GATA-3 autoactivation directs IL-4-independent Th2 development and commitment. Immunity 12:27-37.

Ovington KS, Behm CA (1997) The enigmatic eosinophil: investigation of the biological role of eosinophils in parasitic helminth infection. Mem Inst Oswaldo Cruz 92:93-104.

Ovington KS, McKie K, Matthaei KI, Young IG, Behm CA (1998) Regulation of primary Strongyloides ratti infections in mice: a role for interleukin-5. Immunology 95:488-493.

Pandolfi PP, Roth ME, Karis A, Leonard MW, Dzierzak E, Grosveld FG, Engel JD, Lindenbaum MH (1995) Targeted disruption of the GATA3 gene causes severe abnormalities in the nervous system and in fetal liver haematopoiesis. Nat Genet 11:40-44.

Patton SE, Martin ML, Nelsen LL, Fang X, Mills GB, Bast RC, Jr., Ostrowski MC (1998) Activation of the ras-mitogen-activated protein kinase pathway and phosphorylation of ets-2 at position threonine 72 in human ovarian cancer cell lines. Cancer Res 58:2253-2259.

Pazdrak K, Stafford S, Alam R (1995) The activation of the Jak-STAT 1 signaling pathway by IL-5 in eosinophils. J Immunol 155:397-402.

Pelicci G, Lanfrancone L, Grignani F, McGlade J, Cavallo F, Forni G, Nicoletti I, Pawson T, Pelicci PG (1992) A novel transforming protein (SHC) with an SH2 domain is implicated in mitogenic signal transduction. Cell 70:93-104.

Perlmutter RM (1993) Molecular dissection of lymphocyte signal transduction pathways. Pediatr Res 33:S9-13; discussion S13-15.

Powrie F, Coffman RL (1993) Inhibition of cell-mediated immunity by IL4 and IL10. Res Immunol 144:639-643.

Proudfoot AE, Davies JG, Turcatti G, Wingfield PT (1991) Human interleukin-5 expressed in Escherichia coli: assignment of the disulfide bridges of the purified unglycosylated protein. FEBS Lett 283:61-64.

Proudfoot AE, Fattah D, Kawashima EH, Bernard A, Wingfield PT (1990) Preparation and characterization of human interleukin-5 expressed in recombinant Escherichia coli. Biochem J 270:357-361.

Pugh BF, Tjian R (1990) Mechanism of transcriptional activation by Sp1: evidence for coactivators. Cell 61:1187-1197.

Pugh BF, Tjian R (1991) Transcription from a TATA-less promoter requires a multisubunit TFIID complex. Genes Dev 5:1935-1945.

Punnonen J, Aversa G, Cocks BG, McKenzie AN, Menon S, Zurawski G, de Waal Malefyt R, de Vries JE (1993) Interleukin 13 induces interleukin 4-independent IgG4

and IgE synthesis and CD23 expression by human B cells. Proc Natl Acad Sci U S A 90:3730-3734.

Rabault B, Roussel MF, Quang CT, Ghysdael J (1996) Phosphorylation of Ets1 regulates the complementation of a CSF-1 receptor impaired in mitogenesis. Oncogene 13:877-881.

Raingeaud J, Whitmarsh AJ, Barrett T, Derijard B, Davis RJ (1996) MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen- activated protein kinase signal transduction pathway. Mol Cell Biol 16:1247-1255.

Raingeaud J, Gupta S, Rogers JS, Dickens M, Han J, Ulevitch RJ, Davis RJ (1995) Pro-inflammatory cytokines and environmental stress cause p38 mitogen- activated protein kinase activation by dual phosphorylation on tyrosine and threonine. J Biol Chem 270:7420-7426.

Rana A, Gallo K, Godowski P, Hirai S, Ohno S, Zon L, Kyriakis JM, Avruch J (1996) The mixed lineage kinase SPRK phosphorylates and activates the stress- activated protein kinase activator, SEK-1. J Biol Chem 271:19025-19028.

Ranger AM, Hodge MR, Gravallese EM, Oukka M, Davidson L, Alt FW, de la Brousse FC, Hoey T, Grusby M, Glimcher LH (1998) Delayed lymphoid repopulation with defects in IL-4-driven responses produced by inactivation of NF-ATc. Immunity 8:125-134.

Ransone LJ, Verma IM (1990) Nuclear proto-oncogenes fos and jun. Annu Rev Cell Biol 6:539-557.

Rao A, Luo C, Hogan PG (1997) Transcription factors of the NFAT family: regulation and function. Annu Rev Immunol 15:707-747.

Rao VN, Huebner K, Isobe M, ar-Rushdi A, Croce CM, Reddy ES (1989) elk, tissue-specific ets-related genes on chromosomes X and 14 near translocation breakpoints. Science 244:66-70.

Reddy ES, Rao VN, Papas TS (1987) The erg gene: a human gene related to the ets oncogene. Proc Natl Acad Sci U S A 84:6131-6135.

Reeves R, Nissen MS (1993) Interaction of high mobility group-I (Y) nonhistone proteins with nucleosome core particles. J Biol Chem 268:21137-21146.

Reiner SL, Seder RA (1999) Dealing from the evolutionary pawnshop: how lymphocytes make decisions. Immunity 11:1-10.

Rincon M, Flavell RA (1994) AP-1 transcriptional activity requires both T-cell receptor-mediated and co-stimulatory signals in primary T lymphocytes. Embo J 13:4370-4381.

Rincon M, Flavell RA (1997) T-cell subsets: transcriptional control in the Th1/Th2 decision. Curr Biol 7:R729-732.

Robinson DS, Hamid Q, Ying S, Tsicopoulos A, Barkans J, Bentley AM, Corrigan C, Durham SR, Kay AB (1992) Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. N Engl J Med 326:298-304.

Rolfe FG, Valentine JE, Sewell WA (1997) Cyclosporin A and FK506 reduce interleukin-5 mRNA abundance by inhibiting gene transcription. Am J Respir Cell Mol Biol 17:243-250.

Rolfe FG, Hughes JM, Armour CL, Sewell WA (1992) Inhibition of interleukin-5 gene expression by dexamethasone. Immunology 77:494-499.

Rooney JW, Hoey T, Glimcher LH (1995) Coordinate and cooperative roles for NF-AT and AP-1 in the regulation of the murine IL-4 gene. Immunity 2:473-483.

Rothenberg ME (1998) Eosinophilia. N Engl J Med 338:1592-1600.

Rothenberg ME, Luster AD, Leder P (1995) Murine eotaxin: an eosinophil chemoattractant inducible in endothelial cells and in interleukin 4-induced tumor suppression. Proc Natl Acad Sci U S A 92:8960-8964.

Rothenberg ME, Mishra A, Brandt EB, Hogan SP (2001) Gastrointestinal eosinophils. Immunol Rev 179:139-155.

Rotman HL, Yutanawiboonchai W, Brigandi RA, Leon O, Gleich GJ, Nolan TJ, Schad GA, Abraham D (1996) Strongyloides stercoralis: eosinophil-dependent immune-mediated killing of third stage larvae in BALB/cByJ mice. Exp Parasitol 82:267-278.

Rouse J, Cohen P, Trigon S, Morange M, Alonso-Llamazares A, Zamanillo D, Hunt T, Nebreda AR (1994) A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. Cell 78:1027-1037.

Ryder K, Lanahan A, Perez-Albuerne E, Nathans D (1989) jun-D: a third member of the jun gene family. Proc Natl Acad Sci U S A 86:1500-1503.

Salerno MS, Schwenger GT, Sanderson CJ, Mordvinov VA (2001) Binding of octamer factors to the murine IL-5 CLE0 in primary T-cells and a T-cell line. Cytokine 15:4-9.

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual (2nd ed). New York, Cold Spring Harbour.

Sanderson CJ (1992) Interleukin-5, eosinophils, and disease. Blood 79:3101-3109.

Sanderson CJ, Campbell HD, Young IG (1988) Molecular and cellular biology of eosinophil differentiation factor (interleukin-5) and its effects on human and mouse B cells. Immunol Rev 102:29-50.

Sasaki O, Sugaya H, Ishida K, Yoshimura K (1993) Ablation of eosinophils with anti-IL-5 antibody enhances the survival of intracranial worms of Angiostrongylus cantonensis in the mouse. Parasite Immunol 15:349-354.

Sato S, Katagiri T, Takaki S, Kikuchi Y, Hitoshi Y, Yonehara S, Tsukada S, Kitamura D, Watanabe T, Witte O, et al. (1994) IL-5 receptor-mediated tyrosine phosphorylation of SH2/SH3-containing proteins and activation of Bruton's tyrosine and Janus 2 kinases. J Exp Med 180:2101-2111.

Satoh T, Nakafuku M, Miyajima A, Kaziro Y (1991) Involvement of ras p21 protein in signal-transduction pathways from interleukin 2, interleukin 3, and granulocyte/macrophage colony- stimulating factor, but not from interleukin 4. Proc Natl Acad Sci U S A 88:3314-3318.

Schleimer RP, Sterbinsky SA, Kaiser J, Bickel CA, Klunk DA, Tomioka K, Newman W, Luscinskas FW, Gimbrone MA, Jr., McIntyre BW, et al. (1992) IL-4 induces adherence of human eosinophils and basophils but not neutrophils to endothelium. Association with expression of VCAM-1. J Immunol 148:1086-1092.

Schwenger GT, Mordvinov VA, Karlen S, D'Ercole M, Sanderson CJ (1998) Identification of two novel palindromic regulatory elements in the murine interleukin-5 promoter. Mol Immunol 35:149-158.

Schwenger GT, Fournier R, Hall LM, Sanderson CJ, Mordvinov VA (1999) Nuclear factor of activated T cells and YY1 combine to repress IL-5 expression in a human T-cell line. J Allergy Clin Immunol 104:820-827.

Schwenger GT, Fournier R, Kok CC, Mordvinov VA, Yeoman D, Sanderson CJ (2001) GATA-3 has dual regulatory functions in human IL-5 transcription. J Biol Chem 28:28.

Scott P (1991) IFN-gamma modulates the early development of Th1 and Th2 responses in a murine model of cutaneous leishmaniasis. J Immunol 147:3149-3155.

Sehmi R, Wardlaw AJ, Cromwell O, Kurihara K, Waltmann P, Kay AB (1992) Interleukin-5 selectively enhances the chemotactic response of eosinophils obtained from normal but not eosinophilic subjects. Blood 79:2952-2959.

Shang C, Attema J, Cakouros D, Cockerill PN, Shannon MF (1999) Nuclear factor of activated T cells contributes to the function of the CD28 response region of the granulocyte macrophage-colony stimulating factor promoter. Int Immunol 11:1945-1956.

Shannon MF, Coles LS, Attema J, Diamond P (2001) The role of architectural transcription factors in cytokine gene transcription. J Leukoc Biol 69:21-32.

Sher A, Coffman RL, Hieny S, Scott P, Cheever AW (1990) Interleukin 5 is required for the blood and tissue eosinophilia but not granuloma formation induced by infection with Schistosoma mansoni. Proc Natl Acad Sci U S A 87:61-65.

Shimoda K, van Deursen J, Sangster MY, Sarawar SR, Carson RT, Tripp RA, Chu C, Quelle FW, Nosaka T, Vignali DA, Doherty PC, Grosveld G, Paul WE, Ihle JN (1996) Lack of IL-4-induced Th2 response and IgE class switching in mice with disrupted Stat6 gene. Nature 380:630-633.

Shin EH, Osada Y, Chai JY, Matsumoto N, Takatsu K, Kojima S (1997) Protective roles of eosinophils in Nippostrongylus brasiliensis infection. Int Arch Allergy Immunol 114 Suppl 1:45-50.

Siegel MD, Zhang DH, Ray P, Ray A (1995) Activation of the interleukin-5 promoter by cAMP in murine EL-4 cells requires the GATA-3 and CLE0 elements. J Biol Chem 270:24548-24555.

Simeonovic CJ, Townsend MJ, Wilson JD, McKenzie KU, Ramsay AJ, Matthaei KI, Mann DA, Young IG (1997) Eosinophils are not required for the rejection of neovascularized fetal pig proislet xenografts in mice. J Immunol 158:2490-2499.

Smith MR, DeGudicibus SJ, Stacey DW (1986) Requirement for c-ras proteins during viral oncogene transformation. Nature 320:540-543.

Stein B, Brady H, Yang MX, Young DB, Barbosa MS (1996) Cloning and characterization of MEK6, a novel member of the mitogen- activated protein kinase kinase cascade. J Biol Chem 271:11427-11433.

Stranick KS, Payvandi F, Zambas DN, Umland SP, Egan RW, Billah MM (1995) Transcription of the murine interleukin 5 gene is regulated by multiple promoter elements. J Biol Chem 270:20575-20582.

Stranick KS, Zambas DN, Uss AS, Egan RW, Billah MM, Umland SP (1997) Identification of transcription factor binding sites important in the regulation of the human interleukin-5 gene. J Biol Chem 272:16453-16465.

Stranick KS, Uss AS, Zambas DN, Egan RW, Billah MM, Umland SP (1998) Characterization of the mouse interleukin-5 promoter in a mouse TH2 T cell clone. Biochem Biophys Res Commun 252:56-62.

Su B, Jacinto E, Hibi M, Kallunki T, Karin M, Ben-Neriah Y (1994) JNK is involved in signal integration during costimulation of T lymphocytes. Cell 77:727-736.

Sugaya H, Aoki M, Abe T, Ishida K, Yoshimura K (1997) Cytokine responses in mice infected with Angiostrongylus cantonensis. Parasitol Res 83:10-15.

Sutherland GR, Baker E, Callen DF, Campbell HD, Young IG, Sanderson CJ, Garson OM, Lopez AF, Vadas MA (1988) Interleukin-5 is at 5q31 and is deleted in the 5q- syndrome. Blood 71:1150-1152.

Swan KA, Alberola-Ila J, Gross JA, Appleby MW, Forbush KA, Thomas JF, Perlmutter RM (1995) Involvement of p21ras distinguishes positive and negative selection in thymocytes. Embo J 14:276-285.

Swanson DJ, Zellmer E, Lewis EJ (1998) AP1 proteins mediate the cAMP response of the dopamine beta-hydroxylase gene. J Biol Chem 273:24065-24074.

Takaki S, Kanazawa H, Shiiba M, Takatsu K (1994) A critical cytoplasmic domain of the interleukin-5 (IL-5) receptor alpha chain and its function in IL-5-mediated growth signal transduction. Mol Cell Biol 14:7404-7413.

Takaki S, Murata Y, Kitamura T, Miyajima A, Tominaga A, Takatsu K (1993) Reconstitution of the functional receptors for murine and human interleukin 5. J Exp Med 177:1523-1529.

Takaki S, Tominaga A, Hitoshi Y, Mita S, Sonoda E, Yamaguchi N, Takatsu K (1990) Molecular cloning and expression of the murine interleukin-5 receptor. Embo J 9:4367-4374.

Takaki S, Mita S, Kitamura T, Yonehara S, Yamaguchi N, Tominaga A, Miyajima A, Takatsu K (1991) Identification of the second subunit of the murine interleukin-5 receptor: interleukin-3 receptor-like protein, AIC2B is a component of the high affinity interleukin-5 receptor. Embo J 10:2833-2838.

Takamoto M, Ovington KS, Behm CA, Sugane K, Young IG, Matthaei KI (1997) Eosinophilia, parasite burden and lung damage in Toxocara canis infection in C57Bl/6 mice genetically deficient in IL-5. Immunology 90:511-517.

Takatsu K, Takaki S, Hitoshi Y (1994) Interleukin-5 and its receptor system: implications in the immune system and inflammation. Adv Immunol 57:145-190.

Takeda K, Tanaka T, Shi W, Matsumoto M, Minami M, Kashiwamura S, Nakanishi K, Yoshida N, Kishimoto T, Akira S (1996) Essential role of Stat6 in IL-4 signalling. Nature 380:627-630.

Tan CL (1998) Transcriptional regulation of the mouse interleukin-5 gene in T-lymphocytes. PhD Thesis Australian National University.

Tanese N, Pugh BF, Tjian R (1991) Coactivators for a proline-rich activator purified from the multisubunit human TFIID complex. Genes Dev 5:2212-2224.

Tavernier J, Devos R, Cornelis S, Tuypens T, Van der Heyden J, Fiers W, Plaetinck G (1991) A human high affinity interleukin-5 receptor (IL5R) is composed of an IL5-specific alpha chain and a beta chain shared with the receptor for GM-CSF. Cell 66:1175-1184.

Tavernier J, Tuypens T, Verhee A, Plaetinck G, Devos R, Van der Heyden J, Guisez Y, Oefner C (1995) Identification of receptor-binding domains on human interleukin 5 and design of an interleukin 5-derived receptor antagonist. Proc Natl Acad Sci U S A 92:5194-5198.

Tavernier J, Devos R, Van der Heyden J, Hauquier G, Bauden R, Fache I, Kawashima E, Vandekerckhove J, Contreras R, Fiers W (1989) Expression of human and murine interleukin-5 in eukaryotic systems. DNA 8:491-501.

Thanos D, Maniatis T (1992) The high mobility group protein HMG I(Y) is required for NF-kappa B- dependent virus induction of the human IFN-beta gene. Cell 71:777-789.

Thanos D, Maniatis T (1995) Virus induction of human IFN beta gene expression requires the assembly of an enhanceosome. Cell 83:1091-1100.

Thierfelder WE, van Deursen JM, Yamamoto K, Tripp RA, Sarawar SR, Carson RT, Sangster MY, Vignali DA, Doherty PC, Grosveld GC, Ihle JN (1996) Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. Nature 382:171-174.

Thomas MA, Mordvinov VA, Sanderson CJ (1999) The activity of the human interleukin-5 conserved lymphokine element 0 is regulated by octamer factors in human cells. Eur J Biochem 265:300-307.

Thomas RS, Tymms MJ, McKinlay LH, Shannon MF, Seth A, Kola I (1997) ETS1, NFkappaB and AP1 synergistically transactivate the human GM-CSF promoter. Oncogene 14:2845-2855.

Thompson CB, Wang CY, Ho IC, Bohjanen PR, Petryniak B, June CH, Miesfeldt S, Zhang L, Nabel GJ, Karpinski B, et al. (1992) cis-acting sequences required for inducible interleukin-2 enhancer function bind a novel Ets-related protein, Elf-1. Mol Cell Biol 12:1043-1053.

Timmerman LA, Clipstone NA, Ho SN, Northrop JP, Crabtree GR (1996) Rapid shuttling of NF-AT in discrimination of Ca2+ signals and immunosuppression. Nature 383:837-840.

Ting CN, Olson MC, Barton KP, Leiden JM (1996) Transcription factor GATA-3 is required for development of the T-cell lineage. Nature 384:474-478.

Todd MD, Grusby MJ, Lederer JA, Lacy E, Lichtman AH, Glimcher LH (1993) Transcription of the interleukin 4 gene is regulated by multiple promoter elements. J Exp Med 177:1663-1674.

Tominaga A, Takaki S, Hitoshi Y, Takatsu K (1992) Role of the interleukin 5 receptor system in hematopoiesis: molecular basis for overlapping function of cytokines. Bioessays 14:527-533.

Tominaga A, Takaki S, Koyama N, Katoh S, Matsumoto R, Migita M, Hitoshi Y, Hosoya Y, Yamauchi S, Kanai Y, et al. (1991) Transgenic mice expressing a B cell growth and differentiation factor gene (interleukin 5) develop eosinophilia and autoantibody production. J Exp Med 173:429-437.

Toneguzzo F, Hayday AC, Keating A (1986) Electric field-mediated DNA transfer: transient and stable gene expression in human and mouse lymphoid cells. Mol Cell Biol 6:703-706.

Tong L, Pav S, White DM, Rogers S, Crane KM, Cywin CL, Brown ML, Pargellis CA (1997) A highly specific inhibitor of human p38 MAP kinase binds in the ATP pocket. Nat Struct Biol 4:311-316.

Tournier C, Whitmarsh AJ, Cavanagh J, Barrett T, Davis RJ (1997) Mitogen-activated protein kinase kinase 7 is an activator of the c-Jun NH2-terminal kinase. Proc Natl Acad Sci U S A 94:7337-7342.

Trinchieri G (1993) Interleukin-12 and its role in the generation of TH1 cells. Immunol Today 14:335-338.

Tsai SF, Strauss E, Orkin SH (1991) Functional analysis and in vivo footprinting implicate the erythroid transcription factor GATA-1 as a positive regulator of its own promoter. Genes Dev 5:919-931.

Tsuboi A, Masuda ES, Naito Y, Tokumitsu H, Arai K, Arai N (1994) Calcineurin potentiates activation of the granulocyte-macrophage colony- stimulating factor gene in T cells: involvement of the conserved lymphokine element 0. Mol Biol Cell 5:119-128.

Tsuruta L, Lee HJ, Masuda ES, Yokota T, Arai N, Arai K (1995) Regulation of expression of the IL-2 and IL-5 genes and the role of proteins related to nuclear factor of activated T cells. J Allergy Clin Immunol 96:1126-1135.

Ullrich A, Schlessinger J (1990) Signal transduction by receptors with tyrosine kinase activity. Cell 61:203-212.

Unanue ER, Allen PM (1987) The basis for the immunoregulatory role of macrophages and other accessory cells. Science 236:551-557.

Valentine JE, Sewell WA (1998) Characterisation of inducible DNase I hypersensitive sites flanking the human interleukin-5 gene. Biochim Biophys Acta 1442:218-229.

Vallance BA, Matthaei KI, Sanovic S, Young IG, Collins SM (2000) Interleukin-5 deficient mice exhibit impaired host defence against challenge Trichinella spiralis infections. Parasite Immunol 22:487-492.

Vallance BA, Blennerhassett PA, Deng Y, Matthaei KI, Young IG, Collins SM (1999) IL-5 contributes to worm expulsion and muscle hypercontractility in a primary T. spiralis infection. Am J Physiol 277:G400-408.

van Leeuwen BH, Martinson ME, Webb GC, Young IG (1989) Molecular organization of the cytokine gene cluster, involving the human IL-3, IL-4, IL-5, and GM-CSF genes, on human chromosome 5. Blood 73:1142-1148.

Van Straaten JF, Dokter WH, Stulp BK, Vellenga E (1994) The regulation of interleukin 5 and interleukin 3 gene expression in human T cells. Cytokine 6:229-234.

Walker C, Virchow JC, Jr., Bruijnzeel PL, Blaser K (1991) T cells and asthma. II. Regulation of the eosinophilia of asthma by T cell cytokines. Int Arch Allergy Appl Immunol 94:248-250.

Wang CY, Petryniak B, Thompson CB, Kaelin WG, Leiden JM (1993) Regulation of the Ets-related transcription factor Elf-1 by binding to the retinoblastoma protein. Science 260:1330-1335.

Wang CY, Bassuk AG, Boise LH, Thompson CB, Bravo R, Leiden JM (1994) Activation of the granulocyte-macrophage colony-stimulating factor promoter in T cells requires cooperative binding of Elf-1 and AP-1 transcription factors. Mol Cell Biol 14:1153-1159.

Wang DZ, Ray P, Boothby M (1995) Interleukin 4-inducible phosphorylation of HMG-I(Y) is inhibited by rapamycin. J Biol Chem 270:22924-22932.

Warner LC, Hack N, Egan SE, Goldberg HJ, Weinberg RA, Skorecki KL (1993) RAS is required for epidermal growth factor-stimulated arachidonic acid release in rat-1 fibroblasts. Oncogene 8:3249-3255.

Wasylyk B, Hahn SL, Giovane A (1993) The Ets family of transcription factors. Eur J Biochem 211:7-18.

Wasylyk B, Wasylyk C, Flores P, Begue A, Leprince D, Stehelin D (1990) The c-ets proto-oncogenes encode transcription factors that cooperate with c-Fos and c-Jun for transcriptional activation. Nature 346:191-193.

Wasylyk C, Bradford AP, Gutierrez-Hartmann A, Wasylyk B (1997) Conserved mechanisms of Ras regulation of evolutionary related transcription factors, Ets1 and Pointed P2. Oncogene 14:899-913.

Weiss MJ, Orkin SH (1995) GATA transcription factors: key regulators of hematopoiesis. Exp Hematol 23:99-107.

Wenger RH, Moreau H, Nielsen PJ (1994) A comparison of different promoter, enhancer, and cell type combinations in transient transfections. Anal Biochem 221:416-418.

Wenner CA, Szabo SJ, Murphy KM (1997) Identification of IL-4 promoter elements conferring Th2-restricted expression during T helper cell subset development. J Immunol 158:765-773.

Whitmarsh AJ, Davis RJ (1996) Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. J Mol Med 74:589-607.

Whitmarsh AJ, Cavanagh J, Tournier C, Yasuda J, Davis RJ (1998) A mammalian scaffold complex that selectively mediates MAP kinase activation. Science 281:1671-1674.

Wills-Karp M (2001) IL-12/IL-13 axis in allergic asthma. J Allergy Clin Immunol 107:9-18.

Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL, Donaldson DD (1998) Interleukin-13: central mediator of allergic asthma. Science 282:2258-2261.

Wilson KP, McCaffrey PG, Hsiao K, Pazhanisamy S, Galullo V, Bemis GW, Fitzgibbon MJ, Caron PR, Murcko MA, Su MS (1997) The structural basis for the specificity of pyridinylimidazole inhibitors of p38 MAP kinase. Chem Biol 4:423-431.

Wolffe AP (1994) Nucleosome positioning and modification: chromatin structures that potentiate transcription. Trends Biochem Sci 19:240-244.

Xanthoudakis S, Viola JP, Shaw KT, Luo C, Wallace JD, Bozza PT, Luk DC, Curran T, Rao A (1996) An enhanced immune response in mice lacking the transcription factor NFAT1. Science 272:892-895.

Yamagata T, Mitani K, Ueno H, Kanda Y, Yazaki Y, Hirai H (1997) Triple synergism of human T-lymphotropic virus type 1-encoded tax, GATA- binding protein, and AP-1 is required for constitutive expression of the interleukin-5 gene in adult T-cell leukemia cells. Mol Cell Biol 17:4272-4281.

Yamagata T, Nishida J, Sakai R, Tanaka T, Honda H, Hirano N, Mano H, Yazaki Y, Hirai H (1995) Of the GATA-binding proteins, only GATA-4 selectively regulates the human interleukin-5 gene promoter in interleukin-5-producing cells which express multiple GATA-binding proteins. Mol Cell Biol 15:3830-3839.

Yamaguchi N, Takahashi T, Harada N, Takatsu K (1989) Mechanisms of the interleukin 5-induced differentiation of B cells. J Biochem (Tokyo) 106:837-843.

Yamaguchi Y, Hayashi Y, Sugama Y, Miura Y, Kasahara T, Kitamura S, Torisu M, Mita S, Tominaga A, Takatsu K (1988) Highly purified murine interleukin 5 (IL-5) stimulates eosinophil function and prolongs in vitro survival. IL-5 as an eosinophil chemotactic factor. J Exp Med 167:1737-1742.

Yie J, Liang S, Merika M, Thanos D (1997) Intra- and intermolecular cooperative binding of high-mobility-group protein I(Y) to the beta-interferon promoter. Mol Cell Biol 17:3649-3662.

Yie J, Merika M, Munshi N, Chen G, Thanos D (1999) The role of HMG I(Y) in the assembly and function of the IFN-beta enhanceosome. Embo J 18:3074-3089.

Yoshida T, Ikuta K, Sugaya H, Maki K, Takagi M, Kanazawa H, Sunaga S, Kinashi T, Yoshimura K, Miyazaki J, Takaki S, Takatsu K (1996) Defective B-1 cell development and impaired immunity against Angiostrongylus cantonensis in IL-5R alpha-deficient mice. Immunity 4:483-494.

Young HA, Ghosh P, Ye J, Lederer J, Lichtman A, Gerard JR, Penix L, Wilson CB, Melvin AJ, McGurn ME, et al. (1994) Differentiation of the T helper phenotypes by analysis of the methylation state of the IFN-gamma gene. J Immunol 153:3603-3610.

Young IG, Tan CL, Fisher JA, van Leeuwen BH, Matthaei KI (1999) Regulation of interleukin-5 expression in T lymphocytes: repression by dexamethasone. Interleukin-5

From molecule to drug target for asthma C J Sanderson ed (Marcel Dekker, Inc, New York):pp289-297.

Young PR, McLaughlin MM, Kumar S, Kassis S, Doyle ML, McNulty D, Gallagher TF, Fisher S, McDonnell PC, Carr SA, Huddleston MJ, Seibel G, Porter TG, Livi GP, Adams JL, Lee JC (1997) Pyridinyl imidazole inhibitors of p38 mitogen-activated protein kinase bind in the ATP site. J Biol Chem 272:12116-12121.

Zawel L, Kumar KP, Reinberg D (1995) Recycling of the general transcription factors during RNA polymerase II transcription. Genes Dev 9:1479-1490.

Zerial M, Toschi L, Ryseck RP, Schuermann M, Muller R, Bravo R (1989) The product of a novel growth factor activated gene, fos B, interacts with JUN proteins enhancing their DNA binding activity. Embo J 8:805-813.

Zhang DH, Yang L, Ray A (1998) Differential responsiveness of the IL-5 and IL-4 genes to transcription factor GATA-3. J Immunol 161:3817-3821.

Zhang DH, Cohn L, Ray P, Bottomly K, Ray A (1997) Transcription factor GATA-3 is differentially expressed in murine Th1 and Th2 cells and controls Th2-specific expression of the interleukin-5 gene. J Biol Chem 272:21597-21603.

Zhang DH, Yang L, Cohn L, Parkyn L, Homer R, Ray P, Ray A (1999) Inhibition of allergic inflammation in a murine model of asthma by expression of a dominant-negative mutant of GATA-3. Immunity 11:473-482.

Zheng W, Flavell RA (1997) The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. Cell 89:587-596.

Ziff EB (1990) Transcription factors: a new family gathers at the cAMP response site. Trends Genet 6:69-72.

Zurawski G, de Vries JE (1994) Interleukin 13, an interleukin 4-like cytokine that acts on monocytes and B cells, but not on T cells. Immunol Today 15:19-26.